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The Mechanism by which Retinol Decreases β-Catenin Protein in Retinoic Acid-Resistant Colon Cancer Cells

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The Mechanism by which Retinol Decreases β-Catenin Protein in Retinoic Acid-Resistant Colon Cancer Cells

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Cancer is the second leading cause of death in the United States, following cardiovascular disease. Colorectal cancer is the third leading cause of cancer death in the United States in both men and women (1). The American Cancer Society predicts over 153,000 new colorectal cancer cases and over 52,000 deaths due to colorectal cancer in 2007 (1). Even though the mortality rate has continued to decrease for colorectal cancer in both men and women, there is only a 64% five-year survival rate, unless a distant metastasis is diagnosed, in which case the five-year survival rate drops to only 10%. *All-trans* retinoic acid (ATRA) is currently used as a chemotherapy for acute promyelocytic leukemia and some forms of skin cancer. Unfortunately, chemotherapy with ATRA often results in unpleasant side effects and ATRA-resistant tumors are common due to defects in ATRA signaling. The focus of this study is retinol's effects on β-catenin, a protein essential for colon carcinogenesis. Our goal is to understand the mechanism by which retinol decreases the growth of ATRA-resistant human colon cancer cells. We first determined that retinol, not ATRA, decreases the growth of ATRA-resistant colon cancer cell

lines by slowing cell cycle progression. Next, we examined the effects of retinol treatment on β-catenin, a protein which regulates the transcription of cyclin D1 and c-myc, a protein essential for progress through the cell cycle. We found that retinol significantly decreased β-catenin protein by inducing retinoid X receptor (RXR)α-mediated proteasomal degradation. Lastly, we showed that RXRα directs the degradation of β-catenin by binding β-catenin and transporting it to the proteasome for degradation. Based on these results, we propose a mechanism where retinol increases RXRα protein levels and RXRα-β-catenin binding as well as the movement of β-catenin to the cytosol where it is proteosomally degraded, ultimately leading to slowed cell cycle progression.

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Chapter 1: Introduction and Literature Review

Cancer Statistics

There have been many advances in the understanding and treatment of human diseases in the last few decades and epidemiological data show continuous progress in understanding and diagnosing cardiovascular disease, cancer, cerebrovascular and chronic lower respiratory diseases. Even with the advances made in understanding and treating many diseases, cancer occurrence and survival rates have remained relatively unchanged in over 20 years (1,2). Cancer is the second leading cause of death in the United States, following cardiovascular disease (1). Colorectal cancer is the third leading cause of cancer death in the United States in both men and women, following lung and bronchus, prostate and breast cancer (1). The American Cancer Society predicts over 153,000 new colorectal cancer cases and over 52,000 deaths due to colorectal cancer in 2007. Although the mortality rate has continued to decrease for colorectal cancer in both men and women, there is a 64% 5-year survival rate unless a metastasis is diagnosed. The diagnosis of metastases causes the 5-year survival rate to drop to only 10% (1).

New cells in the colon form at the bottom of the colon crypt. These cells differentiate into functional colon cells as they move up the crypt to the intestinal lumen. Abnormal cells in the colon are believed to also start at the bottom of the colon crypt, but often lack differentiation when they reach the intestinal lumen. The lack of differentiation is a common characteristic of cancer cells. Most often, these abnormal cells are eventually sloughed off into the lumen and excreted. When the abnomal cells divide more rapidly than they are sloughed off, an abnormal mass, or tumor, is produced. A benign tumor is characterized as a slow growing, well differentiated mass of cells that is encapsulated usually in connective tissue. A second type of tumor is a malignant tumor, which has often developed its own blood supply, is fast growing,

poorly differentiated and has broken through the tissue basement membrane in order to metastasize to another tissue. Colon cancer most often metastasizes to the liver. The liver provides a very hospitable environment for the tumor cells to continue growing and because colon cells are attracted to and proliferate in the presence of hepatic growth factor.

Three major factors contribute to the cause of all diseases, including genetics, diet, and the environment. Lifestyle is a modifiable factor contributing to many diseases, while genetics and, in some ways, the environment are unchangeable factors. Lifestyle, specifically diet, is associated with 75-85% of all chronic diseases (3) and changes in diet are a relatively easy way to reduce the risk of many diseases. The most common modifiable factors associated with colorectal cancer include obesity, physical inactivity, smoking, heavy alcohol consumption and a diet high in red or processed meat (1). Surgery and chemotherapy are the most common treatments used for colorectal cancer (1). Oxaliplatin with 5-fluorouracil (5-FU) followed by leucovorin is a common chemotherapy used for patients with metastatic colorectal cancer after surgery (1). Two drugs recently approved by the U.S Food and Drug Administration for the treatment of metastatic colorectal cancer are bevacizumab, which prevents the growth of blood vessels to the tumor and cetuximab, which blocks hormone-like factors that promote cancer cell growth. The major disadvantage to the current chemotherapies is the dangerous side affects. Both oxaliplatin and 5-FU cause myelosuppression and 5-FU also produces mucositis and cardiac toxicity. The two new drugs, bevacizumab and cetuximab, increase risk for arterial thromboembolic events, upper respiratory infections and cause complications in wound healing. Many studies have looked at different compounds in the diet, including vitamin A, vitamin D, dietary fiber and phytochemicals, as potential chemoprevention and chemotherapies for colorectal cancers with less adverse side affects. Vitamin A related drugs have been shown to

have fewer undesirable side affects, including decreased bone mass (4-6), increased cellular differentiation (7), and clinical depression (8). We evaluated the antiproliferative and potentially chemotheraputic effects of vitamin A on human colon cancer cell lines.

Vitamin A

Vitamin A (retinol) is a fat-soluble vitamin that is involved in vision, cellular differentiation, immune function, reproduction and growth. Vitamin A is obtained from the diet from liver, dairy and eggs in the form of retinyl esters and from yellow-orange vegetables and fruits and dark-green leafy vegetables in the form of β-carotene. β-Carotene also has antioxidant capabilities. Vitamin A as retinyl esters is more readily absorbed than β-carotene from the diet (9). Vitamin A in the diet is measured in retinol activity equivalents (RAE). One μg RAE is equal to 1 μg retinol or 12 μg of β-carotene from the diet (10). The dietary reference intake (DRI) of vitamin A for adult males is 900 μg RAE per day (5000 IU) and 700 μg RAE per day (4000 IU) for women (10).

Vitamin A deficiency is rare in the United States, but it is a major problem for the developing world (11). Approximately 127 million preschool-aged children and 7 million pregnant women are vitamin A deficient worldwide (12). Consequences of vitamin A deficiency include: the inability to fight infection, decreased growth, mild to severe stages of xerophthalmia, ultimately resulting in blindness, and increased risk of mortality (12). The most common problem associated with vitamin A deficiency is the inability to fight infection. Vitamin A is essential for an immune response and vitamin A deficiency inhibits the body's ability to mount an attack on an infection often leading to unnecessary death from a normally treatable infection. Globally, 4.4 million preschool children have xerophthalmia and 6 million mothers suffer night blindness during pregnancy (12). Night blindness occurs when the retina does not receive

enough retinol, which the eye converts to retinal, to regenerate the visual pigments bleached by light. Consuming vitamin A will reverse the effects of night blindness. If vitamin A is not administered, total, irreversible blindness will develop.

Vitamin A toxicity is a rare occurrence but can happen after ingesting large amounts vitamin A supplements or retinoid-related drugs, such as Accutane (13-*cis*-retinoic acid). The maximum vitamin A intake for adults is 3000 μg RAE (8000-10000 IU) per day. Vitamin A toxicity has been shown to have adverse side affects. Symptoms of acute vitamin A toxicity from taking too much vitamin A over a short period of time include nausea, vomiting, headaches, altered mental status and muscle and bone tenderness. Symptoms of chronic vitamin A toxicity from supplements or retinoid-related drugs include chapped lips, dry skin, alopecia, abnormal intestinal function leading to constipation and anemia, decreased bone mass, increased cellular differentiation and birth defects when taken during early pregnancy. β-Carotene is not converted to vitamin A efficiently enough to cause vitamin A toxicity, instead it is stored in subcutaneous fat (13).

Discovery

Elmer V. McCollum and M. Davis discovered vitamin A in 1913. A few years later, Thomas Osborne and Lafayette Mendel discovered that butter contained a fat-soluble nutrient, vitamin A. In the early 1930s it was observed that some plant-derived compounds displayed vitamin A activities, which were later named carotenoids. Vitamin A was first synthesized in 1947. Vitamin A is currently used to generically describe compounds that exhibit the biological activity of retinol, the alcoholic form of vitamin A (9). There are many carotenoids that display vitamin A activity and they are termed provitamin A. The term “retinoid” is used to describe

active compounds that are structurally similar to retinol, such as *all-trans* retinoic acid (ATRA) and retinal (9).

Structure

Retinoids contain three distinct structural domains: 1) a β-ionone ring, 2) a polyunsaturated tail and 3) a polar end group. The polar end group exists in several oxidation states, the lowest is retinol and the highest is ATRA (9). Retinoids are hydrophobic, limiting their solubility in water. The multiple double bonds in the chain cause retinoids to be susceptible to photodegradation and oxidation (9).

Absorption and Metabolism

Vitamin A is fat-soluble and is involved in immune function, cellular differentiation, vision, reproduction and growth. As described, the diet contains vitamin A in the form of 1) preformed vitamin A as retinol and retinyl esters in animal-derived food sources and 2) previtamin A carotenoids in plant-derived food sources. Retinyl esters are cleaved within the intestinal lumen to yield retinol (Figure 1.1A). Carotenoids are absorbed by enterocytes and then cleaved to yield retinal, which is subsequently reduced to retinol (Figure 1.1 B). Once absorbed from the gut, retinol is esterified, forming retinyl esters that are packaged for export to the liver via chylomicrons (14). Lipoprotein lipase hydrolyses the retinyl esters contained in the chylomicrons, providing most cells with access to free retinol (15). Retinol is excreted from the liver bound to retinol binding protein (RBP). RBP is bound to transthyretin. Some free retinol also exists in the circulation (16). Because of retinol's poor solubility in water, it dissolves in the hydrophobic core of the cellular membrane suggesting retinol does not require a receptor to enter into a cell (9). Retinol enters the hydrophilic environment of the cytosol bound to cellular retinol binding protein (CRBP) I or II (Figure 1.2). CRBP I is expressed in all tissues and facilitates

retinol uptake into the target cell and directs the intracellular metabolism of retinol to retinyl esters, its storage form or to an active metabolite, such as ATRA (14,17,18). In contrast, CRBP II is expressed only in the villi of enterocytes where it directs the metabolism of retinol to retinyl esters for chylomicron export (17).

In most cells, retinol is either esterified for storage to retinyl esters or metabolized to ATRA (Figure 1.2). ATRA transport also requires binding proteins, specifically, the cellular retinoic acid binding proteins (CRABP) I and II. CRAPBs solubilize and protect ATRA in the cytosol and act as transporters, moving ATRA to various cellular components. Both CRABP I and II are present in the cytosol and nucleus. In the adult, CRABP I is expressed in all tissues, but CRABP II is expressed only in the skin, uterus, ovary, and choroid plexus, some cholinergic neurons and the pia mater (19,20). CRABP I appears to decrease cellular responses to ATRA by catalyzing its degradation and lowering active intracellular ATRA concentrations (21). In contrast, CRABP II appears to sensitize cells to the effects of ATRA by delivering ATRA to RAR increasing ATRA-mediated gene transcription (19,22). Alternatively, retinol can also be converted to 4-oxoretinol (23,24) and the retro-retinoids, 14-hydroxy-4,14-retro-retinol (HRR) (25) and anhydroretinol (AR) (26) (Figure 1.2), which are also bioactive metabolites of retinol. The types and amounts of biologically active metabolites made from retinol vary with cell type and differentiation state.

Function

The actions of retinol's metabolites are generally mediated by nuclear retinoid receptors, RARα, β and γ and retinoid X receptor (RXR) α, β and γ. The conventional pathway of retinol metabolism relies on ATRA to be transported to the nucleus by CRABP II, where it exerts its effects on cell growth and differentiation by binding RAR. In the absence of ATRA, a

corepressor is bound to the RAR, preventing gene transcription via retinoic acid response elements (RARE). When ATRA binds the RAR, the corepressor is released and replaced with a coactivator (Figure 1.3). These corepressors and coactivators mediate their effects by recruiting proteins that modulate the acetylation of histones surrounding the DNA containing the RARE. Thus, RAR corepressor proteins recruit histone deacetylase complexes (HDACs) to remove the acetyl groups from the lysine residues of histones and prevent gene transcription. In contrast, RAR coactivator proteins recruit histone acetyl transferases (HATs) to acetylate these lysine residues, unwind DNA and facilitate gene transcription (Figure 1.3) [reviewed by (27)]. The major RAR corepressors are nuclear receptor corepressor (N-CoR) and silencing mediator for retinoid and thyroid hormone receptors (SMRT) (28,29). These receptors do not interact with ligand bound RAR, but bind, along with Sin3, to unliganded RAR and recruit HDACs to repress transcription (Figure 1.3). Upon ATRA binding, the ligand-binding domain of the RAR changes shape and releases the corepressors. Coactivators are then recruited to the RAR. RAR heterodimerize with RXR and bind to RARE. RARE are usually composed of direct repeats of the consensus half-site sequence AGGTCA separated by five nucleotides and located in the 5' regulatory regions of retinoid-responsive genes (Figure 1.2). When ATRA binds to the RAR member of the RAR/RXR heterodimer, gene transcription via RARE is induced [for review see: (30)]. Interestingly, ATRA induces the expression of the RAR through this mechanism, sensitizing the cell to the growth inhibitory effects of ATRA (31-33).

Retinoids & Cancer

Retinoids have been shown to suppress carcinogenesis in a variety of tissue types, including oral, skin, bladder, lung, prostate, breast and colon cancers in experimental animals (34-38). Clinically, retinoids are able to reverse premalignant skin lesions and inhibit the

development of primary tumors in the head and neck area and in xeroderma pigmentosum patients (39). Therefore, retinoids could be useful in both chemotherapy and chemoprevention of human cancers. Several *in vivo* studies have examined the chemopreventive effects of retinoids in a rat model of carcinogen-induced colon cancer. These studies indicate that retinol, *9-cis*-retinoic acid (9-*cis*-RA), and 4-(hydroxyphenyl)retinamide (4-HPR) can inhibit the formation of aberrant crypt foci, a precursor to colon cancer (34,38,40,41). *9-Cis*-RA and 4-HPR can also decrease the number of carcinogen-induced colon tumors in rats (38). Retinyl palmitate was also recently shown to inhibit high fat diet-induced aberrant crypt foci in rats (34). Additionally, several *in vitro* studies indicate that retinoids have antiproliferative effects on colon cancer cell lines and may hold potential for both chemoprevention and chemotherapy of colon cancer (42-49). Retinoids have also been shown to inhibit metastasis in a variety of model systems. For example, dietary retinyl palmitate decreased malignant melanoma metastasis in mice (50). ATRA decreased breast cancer (51), gastric cancer (52) and colon cancer cell invasion (53) *in vivo*. Also retinol decreased hepatic metastases in a hamster model of pancreatic ductal carcinoma (54). With their chemopreventative ability and potent antiproliferative effects on cancer cells *in vitro*, retinoids may have potential for both chemoprevention and chemotherapy of colon cancer.

Retinoic Acid Resistance

Traditionally, all the effects of retinoids were believed to be mediated via the ATRA/RAR/RXR/RARE pathway. As mentioned, ATRA is not the only bioactive metabolite of retinol and the diet contains very little ATRA (14). ATRA resistance is also a common and often spontaneous phenomenon (42,46,55-58). ATRA resistance is defined as the inability of ATRA to inhibit cell growth and/or induce differentiation. Defects in retinoid metabolism and signaling

resulting in ATRA resistance are found in many types of cancers and tumor-derived cell lines (45,46,56,58-60). ATRA-resistance is believed to be caused by a defect in RAR α, β, or γ induction in response to ATRA (42,45,46,56) due to methylation. The disrupted receptor varies with cell line.

As mentioned, the diet contains very little ATRA. The intestinal lumen, including colonocytes, is primarily exposed to retinol. Once absorbed, retinol is esterified, forming retinyl esters that are packaged for export to the liver via chylomicrons (14). The colon and liver are exposed to higher levels of retinol compared to other tissues in the body due to absorption of retinol in the gut and the storage of retinol in the liver. Serum retinol levels in non-vitamin A deficient animals vary from 1-2 μM, regardless of supplementation status, [for a review please see: (61)]. Hepatic retinol concentrations increase with dietary vitamin A supplementation and values of > 90 μM have been reported (62). The intraluminal concentrations of retinol in the colon have not been measured to our knowledge. Taken together, it is more relevant to examine the effects of retinol on colon cancer cell growth because colon cells are primarily exposed to retinol and ATRA resistance is common in colon carcinomas.

Growth Inhibition

Cell division and death are essential functions in the normal development and maintenance of eukaryote cells. Disorders in cell division or cell death can result in aberrant embryogenesis, neurodegenerative disorders, or cancer (63). Slowing the division or causing the death of cancer cells leads to smaller, less aggressive tumors. There are four ways to inhibit tumor growth: apoptosis, necrosis, cellular differentiation and cell cycle arrest.

<u>Apoptosis</u>

Apoptosis is a type of programmed cell death characterized by morphological changes such as cell shrinkage, chromatin condensation and breakdown of the cell into small fragments (64). Apoptosis can be initiated by two pathways involving either the release of cytochrome c or the activation of death receptors (65,66). Loss of control of programmed cell death leads to apoptosis resistance and can accelerate the invasion and metastasis of tumor cells (67). Chemotherapy and irradiation exert their affects by inducing apoptotic pathways. Resistance to chemotherapy generally coincides with resistance to apoptosis.

Necrosis

Necrosis is cell death caused by injury, infection, inflammation and hypoxia. The release of enzymes stored by the lysosome triggers the digestion of cellular compartments or of the cell itself causing necrosis (68). Unlike apoptosis, which is controlled, programmed cell death, necrotic cells frequently release harmful chemicals that damage surrounding cells (68).

Cellular Differentiation

Through cellular differentiation cell physiology, including: size, shape, polarity, metabolic activity and responsiveness to signals may change. Progression of cancer is judged by changes in organization, differentiation, proliferation and invasion (69). The more differentiated the cell, the closer it remains to performing normal cellular functions. Less differentiated cells often defy classification and the cell has lost most of its ability to function as a differentiated cell. For example, if cells in the colon become undifferentiated they lose the ability to regulate growth and death signals causing uncontrolled undifferentiated colon cell growth. Undifferentiated colon cancer cells proliferate without restriction increasing the chance of mutation, invasion and eventually metastasis of the undifferentiated colon cell.

Cell Cycle Arrest

The cell cycle consists of four phases: $G_{0/1}$, S, G_2 and M. Most cells are in G_0, the quiescent stage, while not dividing. With tightly controlled regulation, cells enter the G_1 phase due to an increase in cyclin D1 protein (70). Cyclin D1 and cyclin dependent kinases (cdk)-4 and -6 phosphorylate retinoblastoma protein (pRb), which releases E2F. Upon release, E2F increases the transcription of cyclins A and E which push the cell into the S, or DNA synthesis phase. The cell continues through the cell cycle until the M phase where cells physically divide creating a new cell. Many proteins tightly control the division of cells, in particular p53. p53 is the most frequently mutated tumor suppressor gene in all cancers and without its constant control of the cell cycle, cancer cells are able to divide without regulation (70).

β-Catenin

β-Catenin protein was first discovered in humans as part of the epithelial (E)-cadherin membrane complex (71). The function of β-catenin and its role in cell signaling was later elucidated (72,73). β-Catenin is found in three locations within the cell: 1) the membrane-bound adherens complex, where it functions in cell to cell adhesion, 2) the nucleus, where it stimulates gene transcription, and 3) the cytoplasm where it serves as a pool for translocation to the membrane or nucleus, or it can be targeted for degradation (Figure 1.3).

E-Cadherin and β-catenin, along with α- and γ-catenin, are essential to the adherens complex, which controls normal tissue morphogenesis, including segregation of cell types, support structures, and differentiation (74). E-Cadherin forms the functional component of the adherens junction between cells in epithelial tissue (75). The E-cadherin complex is embedded in the membrane and β-catenin binds directly to the tail of E-cadherin (76). An intact E-cadherin-catenin complex is required for maintenance of normal intracellular adhesion (75).

Cytosolic β-catenin can be degraded by the proteasome or translocated to the nucleus. The transcriptional function of β-catenin is mediated through its interaction with the T cell factor/lymphoid enhancer factor (TCF/LEF) transcription complex in the nucleus (72,73) (Figure 1.4). Stable β-catenin accumulating in the cytosol, freely enters the nucleus where it interacts with the TCF/LEF transcription factors, forming an active transcription complex. These complexes regulate the transcription of a variety of genes involved in cell proliferation including cyclin D1 (77) and c-myc (78). They also regulate genes involved in tumor progression and metastasis including matrilysin (79) and cyclooxygenase-2 (cox-2) (80).

Cyclin D1 is an important regulator of the cell cycle and ultimately cell proliferation. It is required for the cell to progress from the $G_{0/1}$ phase into the S phase (70). It has been known for some time that cyclin D1 protein is overexpressed in colon cancers but genetic mutations in cyclin D1 are rarely found in colon carcinomas (81,82). Tetsu and McCormick (77) have shown that both wild-type and mutant forms of β-catenin can increase the transcription of cyclin D1. This is due to β-catenin/TCF/LEF complexes binding to the cyclin D1 promoter. The promotion of cyclin D1 expression by β-catenin was also inhibited by transfection with wild-type adenomatous polyposis coli (APC), which reduced nuclear β-catenin levels (83). Wong and Pignatelli have shown, *in vivo*, that cyclin D1 overexpression only takes place in association with free nuclear β-catenin in human colorectal cancers (84).

c-Myc levels in the normal cell are tightly regulated by growth factors and extracellular contacts (70). There is very little c-myc expression when the cell is in a resting state, but with proper stimulation c-myc is expressed at the beginning of the cell cycle and persists throughout the cell cycle until the cell returns to a quiescent state (70). Cancer cells with high expression of c-myc show increased cell proliferation by accelerating cells through the G_1 and S phases of the

cell cycle, evading cell cycle checkpoints (70). It has been shown that both wild-type and phosphorylation-resistant β-catenin protein increases c-myc gene expression via TCF/LEF binding to the c-myc promoter (77). Thus, increases in nuclear β-catenin lead to an increase in c-myc expression and ultimately uncontrolled cell growth.

Matrilysin (also known as MMP-7) is a matrix metalloproteinase (MMP). MMPs are a family of zinc-dependent proteases that have the ability to degrade components of the extracellular matrix and have been implicated in tumor invasion (53). MMPs are often active during tumor invasion, resulting in increased proteolytic degradation of the extracellular matrix. Upon activation, matrilysin exhibits proteolytic activity against a variety of extracellular matrix substrates, including collagens, proteoglycans, elastin, laminin, fibronectin, and casein (85,86). Matrilysin is expressed in approximately 90% of colorectal cancers and is thought to be important in mediating stromal invasion (87). The matrilysin gene promoter has at least two TCF-binding sites and the β-catenin/TCF/LEF complex has been shown to increase matrilysin promoter activity *in vitro* (78).

β-Catenin & Colon Cancer

β-Catenin plays a crucial role in the development of colorectal cancer. Mutations in β-catenin degradation pathways, which are present in 70-80% of colorectal tumors, lead to elevated nuclear β-catenin and an increase in cell proliferation genes (73,84,88,89). APC and β-catenin are the primary targets for mutation and these mutations are regarded as critical for colon carcinogenesis (84,89). Inactivation of APC is believed to be the first event in colon tumorigenesis (88). The most common mutations found in the gene encoding for β-catenin affect the protein's N-terminal serine (Ser) and threonine (Thr) residues that are required for phosphorylation and ubiquitin-mediated degradation. Without the ability to phosphorylate β-

catenin, the glycogen synthase kinase (GSK)-3β/APC degradation pathway cannot decrease β-catenin protein leading to increased β-catenin in the nucleus and increased transcription of cell proliferation and metastasis genes. However, two other pathways to degrade β-catenin exist; the p53 and RXR-mediated pathways.

p53 is mutated in 50% of all cancers (90). Mutations in p53 generally lead to the inactivation of its tumor suppressor function. Losing the p53-regulation of the cell cycle leads to an increase in genes involved in cell proliferation, such as cyclin D1 and c-myc. As described above, increased cyclin D1 is required for cells to go into the S phase of the cell cycle, which is tightly regulated by p53. Without the ability to regulate the cell cycle due to mutated p53, cancer cells are able to constantly move into the S phase and through the cell cycle uncontrollably.

The RXR protein also plays a critical role in decreasing β-catenin protein. There are three isoforms of RXRα, β and γ and each isoform has 3 subtypes, 1, 2 and 3. Because p53 mutations are incredibly common in cancer and β-catenin and APC mutations are almost certain in colon cancers, some cells may fully rely on the RXR-mediated β-catenin degradation pathway to control β-catenin-regulated cell proliferation. In our evaluation of colon cancer cell growth, we will focus on a potential chemotherapy for human colon cancer cells that contain mutant β-catenin, mutant p53 or mutant p53 and APC null.

β-Catenin Degradation

As mentioned above, β-catenin degradation is controlled by three pathways: the GSK-3β/APC, p53/Siah-1/APC and the RXR-dependent degradation pathway (Figure 1.5). In the cytosol, β-catenin degradation involves interaction with APC, AXIN and GSK-3β (72,73) (Figure 1.4). GSK-3β is responsible for phosphorylating Ser45, Thr41, Ser37, and Ser33 residues on the N-terminus of β-catenin. Phosphorylation of the Ser and Thr residues is essential

to target the protein for ubiquitination and proteasomal degradation (84). APC directly binds β-catenin, acting as a scaffolding protein, enhancing the phosphorylation of β-catenin by GSK-3β; therefore APC is regarded as a promoter of β-catenin degradation and is thought to be needed for efficient degradation of the protein (89). Phosphorylated β-catenin interacts with the F box protein β-TrCP (91,92). The F box protein contains specific protein recognition sites and most F box proteins recognize phosphorylated proteins. β-TrCP binds phosphorylated β-catenin, which is then tagged with multiple copies of the ubiquitin protein targeting it for proteasomal degradation (Figure 1.6). GSK-3β is regulated by the Wnt family. The binding of Wnt to its trans-membrane receptor, Fz, leads to an increase in Dishevelled (Dvl) activity, inhibiting GSK-3β phosphorylation activity (84). GSK-3β is also regulated by Akt. Active, phosphorylated Akt phosphorylates GSK-3β rendering it inactive. Without the ability to phosphorylate β-catenin, it accumulates in the cytosol and translocates into the nucleus where it can transcribe cell proliferation and metastasis genes.

β-Catenin degradation also occurs through two alternative pathways, the p53/Siah-1/APC pathway and the RXR-dependent pathway (Figure 1.5). Siah-1 expression is inducible by p53 and overexpression of Siah-1 inhibits cell proliferation, promotes apoptosis, and suppresses tumor formation (93,94). Siah-1 binds Ebi, another F box protein that binds β-catenin but unlike β-TrCP, Ebi binds β-catenin independent of the phosphorylation sites recognized by β-TrCP (Figure 1.6) (95). APC also enhances the efficiency of this pathway by again acting as a type of scaffolding structure holding Ebi and β-catenin in close proximity. With β-catenin and Ebi bound to APC, β-catenin can be targeted for ubiquitin-dependent proteolysis (94). The exact mechanism of how Ebi targets β-catenin for degradation is still under investigation.

The third pathway that mediates β-catenin degradation is the RXR-dependent pathway. RXR agonists have been shown to inactivate β-catenin mediated transcription and induce β-catenin degradation through endogenous and transfected RXR in kidney adenovirus cells (96). Decreases in β-catenin protein were dependent on an RXR-mediated proteasomal degradation pathway, which is independent of RXR-mediated gene transcription (96). Most importantly, the RXR-regulated β-catenin degradation was independent of the p53/Siah/APC and GSK-3β/APC pathways in kidney adenovirus cells (96). The RXR-dependent pathway decreases free β-catenin by inducing β-catenin degradation in cells with loss-of-function mutations in APC and p53 as well as in N-terminal-mutated phosphorylation-resistant β-catenin (96). Our work shows a direct interaction between RXRα and β-catenin proteins leading to increased proteasomal degradation in human colon cancer cell lines.

Proteasomal Degradation.

Regardless of degradation pathway, it is believed that β-catenin protein turnover is directed by the proteasome. The proteasome is a large multiprotein organelle that contains proteases responsible for intracellular protein degradation in eukaryotic cells (97). The proteasome is composed of two functional components: a 20S core catalytic complex and a 19S regulatory subunit (98). Proteins that are to be degraded are tagged with ubiquitin chains, which bind to a receptor on the 19S complex (97). Once the 19S subunit binds to the ubiquitinated protein, the 19S subunit de-ubiquitinates and unfolds the protein in order to shuttle it into the 20S core complex. The 20S core complex is made up of four stacked, multiprotein rings (97). The outer α subunit rings form a narrow channel that allows only denatured proteins to enter the catalytic compartment formed by the central β subunit rings (99-101). The β subunit rings degrade the protein, reducing it to small polypeptides three to 22 residues in length (102).

Proteasomes customarily degrade proteins tagged with ubiquitin, but proteasomes can degrade proteins by ubiquitin-independent mechanisms as well (103,104). Proteolysis by the proteasome is a fundamental metabolic process within the cell and recognition of misfolded and abnormal proteins is essential for cell survival regardless of ubiquitin tagging (102).

Lysosomal Protein Degradation.

Lysosomes also degrade proteins. The lysosome is most often thought to destroy bacteria and "worn out" organelles but the lysosome also carries enzymes to degrade nucleotides, proteins, lipids, and phospholipids. During lysosomal degradation, the protein is enclosed in a vacuole, which fuses with a vesicle containing lysosomal enzymes or hydrolases, forming the lysosome. After the synthesis of the lysosome, the pH becomes more acidic due to a hydrogen ion ATPase located in the membrane of the lysosome that acidifies the lysosomal environment. This decrease in pH activates the hydrolases which degrades the protein.

Nuclear Receptors and β-Catenin Regulation

Nuclear receptors are involved in transport and degradation of β-catenin protein. The effect of nuclear receptors on β-catenin protein and β-catenin-TCF/LEF transcriptional activity varies with receptor type. In general, nuclear receptors, including RAR, RXR, vitamin D receptors (VDR), androgen receptors and peroxisome proliferators-activated receptors γ (PPARγ), interact with β-catenin to directly or indirectly reduce β-catenin-TCF/LEF-mediated gene transcription. Nuclear receptors can bind β-catenin, sequestering it away from the TCF/LEF complex, directly decreasing β-catenin-TCF/LEF-mediated gene transcription. Nuclear receptors are also capable of binding to and shuttling β-catenin out of the nucleus and relocating it to the membrane or transporting β-catenin to the proteasome for degradation. For example, Easwaran, et. al. (105) showed RAR binds β-catenin *in vitro* and blocks β-catenin

mediated gene transcription *in vivo*. Many studies have also shown that androgen receptors bind directly to β-catenin inhibiting β-catenin-TCF/LEF-mediated gene transcription, ultimately reducing cell growth in prostate cells (106-109). Furthermore, RXRα and PPARγ have been shown to directly interact with β-catenin leading to a decrease in TCF/LEF transcriptional activity in malignant prostate cells (110). The VDR exhibit both direct and indirect interference in β-catenin-TCF/LEF-mediated gene transcription. VDR binds with β-catenin to block its binding with TCF/LEF, directly inhibiting the expression of β-catenin-TCF/LEF genes in colon carcinoma cells (111). VDR also export β-catenin from the nucleus and relocate it to the plasma membrane (111), indirectly decreasing β-catenin-TCF/LEF-mediated gene transcription. *In vivo,* PPARγ have been shown to interact with β-catenin in cells containing a functional APC/Axin complex (112) and β-catenin bound to activated PPARγ induces β-catenin proteasomal degradation in mouse fibroblasts indirectly reducing β-catenin-TCF/LEF-mediated gene transcription (113). RXR also indirectly decreases β-catenin-mediated gene transcription by inducing the proteasomal degradation of β-catenin in several cell lines including APC-null and p53-mutant colon cancer cell lines (96). The data suggest that interaction between nuclear receptors and β-catenin reduces β-catenin-TCF/LEF-mediated gene transcription, ultimately altering cell cycle progression and decreasing cell growth.

Retinoids & β-Catenin

Several studies show that the signaling activity of β-catenin is altered in the presence of retinoids and/or retinoid receptors. In general, retinoid receptors either inhibit β-catenin-mediated gene transcription, as in the case of RAR or decrease β-catenin protein levels, as in the case of RXR. For example, RXR decrease β-catenin mediated gene transcription by inducing the proteasomal degradation of β-catenin in several cell lines including APC-null and p53-mutant

colon cancer cell lines (96). RXRα and PPARγ have been shown to directly interact with β-catenin leading to a decrease in TCF/LEF transcriptional activity in malignant prostate cells (110). In contrast, SKBR3 breast cancer cells, which express low endogenous levels of β-catenin (114,115), exhibit increased β-catenin levels due to enhanced β-catenin protein stability when treated with 9-*cis*-RA, a ligand for both RAR and RXR (116). β-catenin levels were not affected by 9-*cis*-RA treatment in the MCF7 breast cancer cell line (105), but 9-*cis*-RA treatment did decrease β-catenin/TCF/LEF mediated gene transcription in MCF7, CaCo-2, HS578t and SKBR3 cells (105). An additional study by the same group showed that 9-*cis*-RA treatment reduced cytoplasmic levels of exogenously expressed β-catenin in SKBR3 cells by targeting β-catenin to the cell membrane (117). Also, the transcription of a cyclin D1 reporter construct lacking TCF binding sites was decreased by 9-*cis*-RA in SKBR3 cells, indicating that AP-1 mediated cyclin D1 transcription in this cell line. In contrast to the SKBR3 cell line, 9-*cis*-RA treatment decreased the transcription of a wild type (wt) cyclin D1 reporter construct, containing TCF binding sites, in the SW480 colon cancer cell line, which expresses high levels of β-catenin. However, 9-*cis*-RA did not alter the transcription of a cyclin D1 reporter construct lacking TCF binding sites, indicating that β-catenin/TCF/LEF complex regulates cyclin D1 transcription in SW480 colon adenoma cells. The effects of 9-*cis*-RA on β-catenin/TCF/LEF-mediated gene transcription are due to the ability of ligand-bound RAR to compete with TCF for β-catenin binding. This competition prevents β-catenin-mediated gene transcription (105). A known RXR ligand, 9-cis-RA is not found in the diet and, although controversial, it is not found at physiologically relevant levels in the body. Previous studies also showed that RXRα protein interacted with β-catenin*, in vitro*, inducing β-catenin degradation and that these actions were enhanced by but did not require the presence of a RXR agonist (96,110). Retinol, which is not

an RXR ligand, is in the diet and continuously in the circulation and therefore, is the more relevant retinoid to treat colon cancer cells.

Cell Lines

The main cell lines used in these studies are HCT-116, SW620 and WiDr. The HCT-116 cell line is a colorectal carcinoma (56) that expresses wild type (wt) p53 and APC, but was heterozygous for phosphorylation-resistant β-catenin (deletion of codon 45: $CTNNB1^{WT/\Delta 45}$) (118). The SW620 cell line is a colorectal adenocarcinoma (42) that expresses wt β-catenin, mutant p53 (R273H), and is APC null (119). The WiDr cell line is also a colorectal adenocarcinoma (120) that contains wt APC and β-catenin but mutant p53 (R273H) (121). The HCT-15 cell line was used in the beginning of the study as a positive control because the HCT-15 cells are ATRA-sensitive human colorectal adenoma cells (122).

Summary

β-Catenin and APC are mutated in 70-80% of colorectal tumors. Mutations in β-catenin prevent its degradation leading to increased free nuclear β-catenin and uncontrolled cell growth. In normal colonocytes, APC aids in the degradation of β-catenin. Thus, mutated, non-functional APC also leads to nuclear accumulation of β-catenin. Retinoids and retinoid receptors can alter β-catenin signaling through many different processes, including increasing β-catenin degradation, sequestering β-catenin away from TCF/LEF transcription complex or shuttling β-catenin to the cellular membrane increasing cell stability. Because ATRA resistance occurs spontaneously in many types of cancer cells and human colonocytes are exposed primarily to retinol via the intestinal lumen, retinol may prove more effective in treating colon cancers, including those colon cancer cells that have become ATRA-resistant.

This dissertation focuses on the study of retinol as an inhibitor of ATRA-resistant human colon cancer cell line growth and the mechanism by which retinol decreases β-catenin protein *in vitro*. The aim of this chapter was to provide background information concerning retinoid biology, the role of β-catenin in colon cancer, and β-catenin degradation pathways. Chapter 2 will focus on the initial studies showing the mechanism of growth inhibition in ATRA-resistance colon cancer cells when treated with retinol. Chapter 3 will build on these findings and examine the effect of retinol on total cellular β-catenin levels in ATRA-resistant human colon cancer cell lines, as well as the specific pathway involved in retinol-induced β-catenin protein degradation. Chapter 4 will focus on the RXRα protein and the mechanism in which it increases the degradation of β-catenin. Together, these studies suggest that retinol can inhibit the growth of ATRA-resistant human colon cancer cells *in vitro* by increasing RXRα-mediated β-catenin degradation leading to a decrease in cell cycle progression proteins and ultimately slowing the growth of human colon cancer cells. Chapter 5 will summarize the findings in these studies and suggest a future direction to continue to understand the growth inhibitory effects of retinol on ATRA-resistant human colon cancer cell lines.

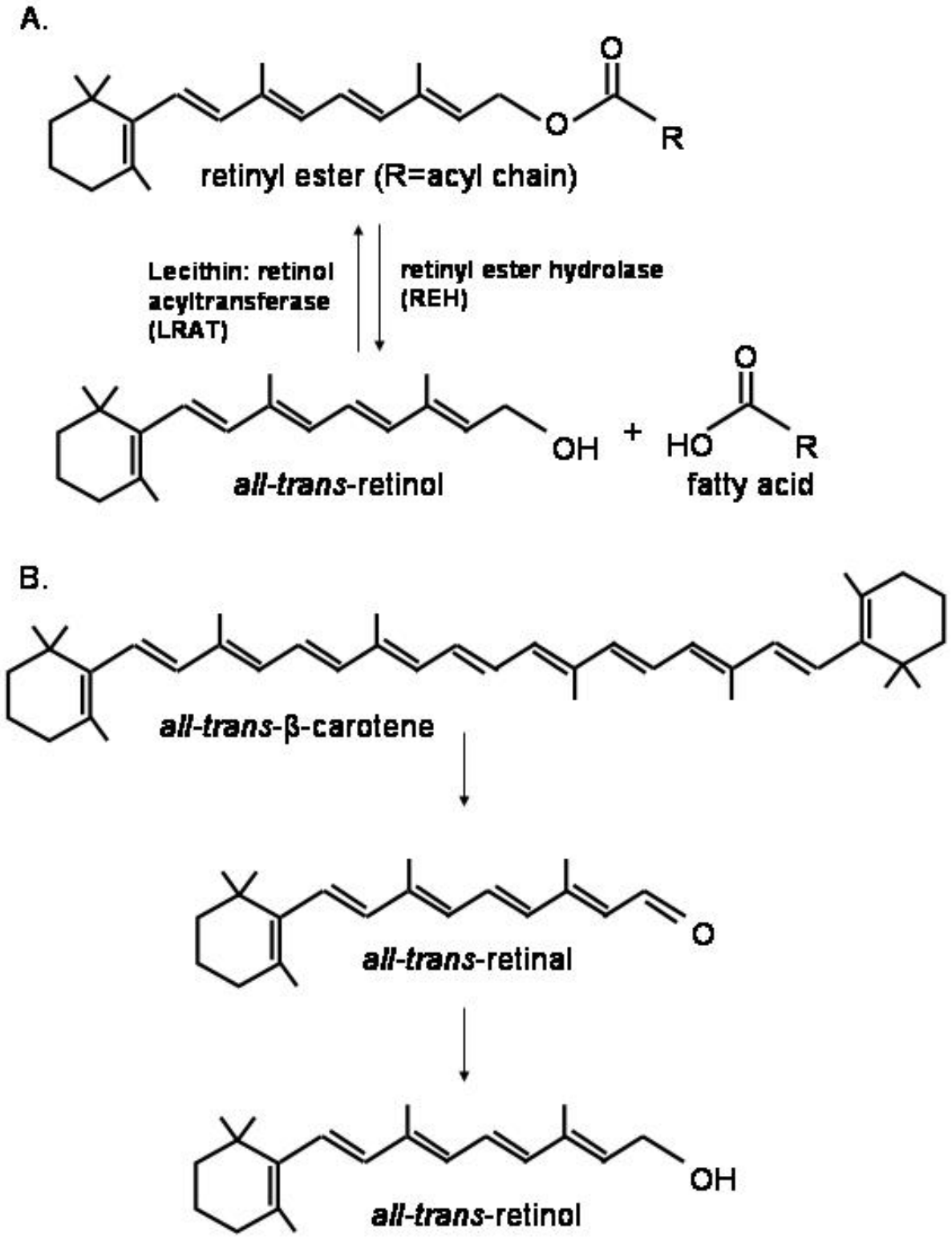

Figure 1.1 Retinyl esters (A) and β-carotene (B) are hydrolyzed in the intestinal luman to yield retinol. Adapted from (9).

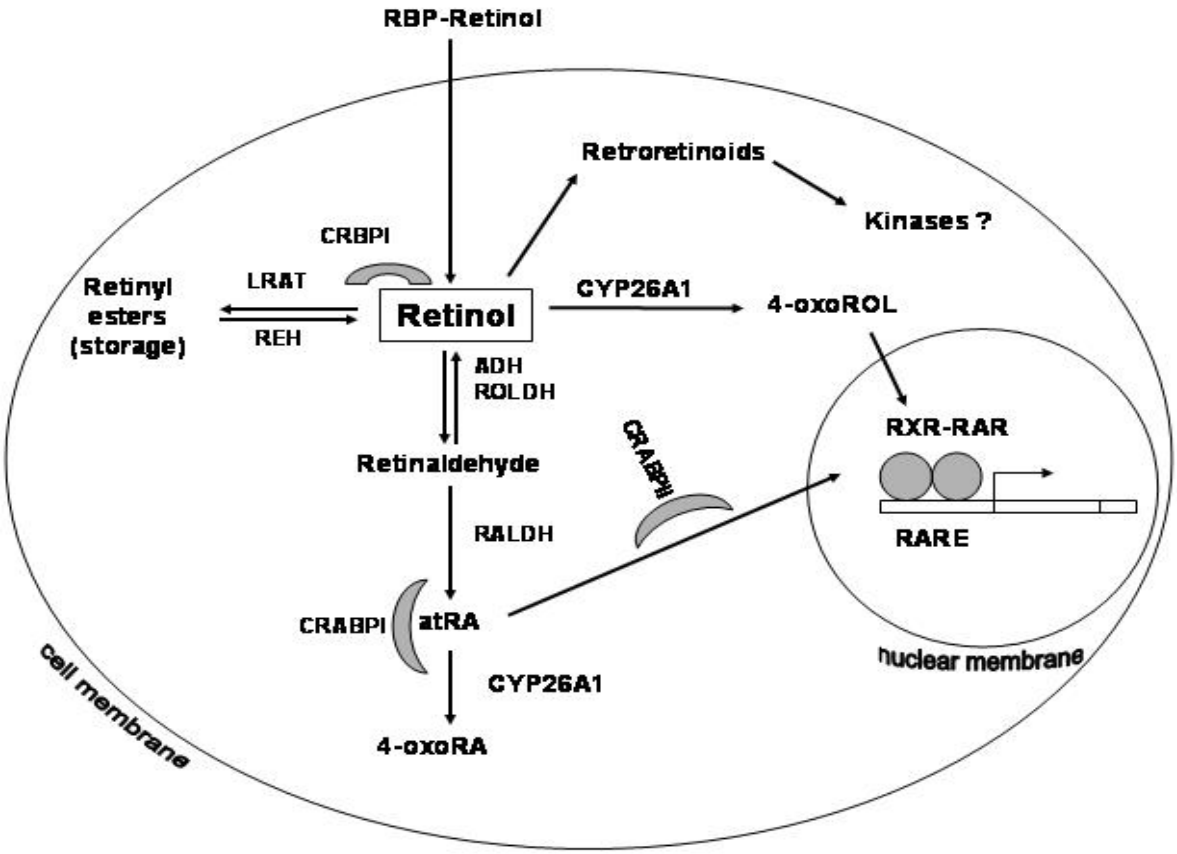

Figure 1.2 Intracellular retinol metabolism and signaling via RAR, RXR and RARE. Abbreviations are as follows: RBP, retinol binding protein; LRAT, lethicin:retinol acyltransferase; REH, retinyl ester hydrolase; ADH, alcohol dehydrogenase; ROLDH, retinol dehydrogenase; RALDH, retinal dehydrogenase; RA, retinoic acid; 4-oxoRA, 4-oxoretinoic acid; 4-oxoROL, 4-oxoretinol; AR, anhydroretinol; 14-HRR, 14-hydroxy-4,14*retro*-retinol.

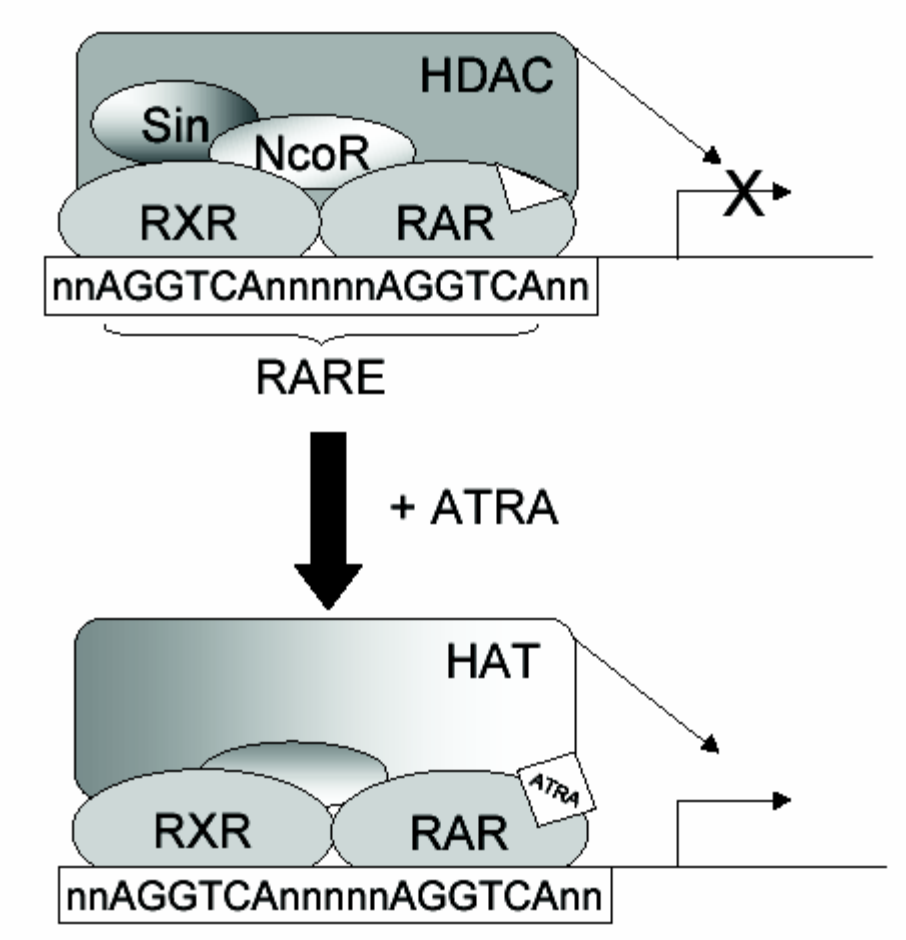

Figure 1.3 All-trans-retinoic acid (ATRA) regulates gene transcription via retinoic acid receptors and/or retinoid X receptors (RAR–RXR) bound to retinoic acid response elements (RARE). RAR and RXR bind to RAREs. Unliganded RARs are complexed with histone deacetylase complexes (HDAC), inhibiting gene transcription. Upon ATRA binding, the HDAC and coregulator proteins are released and new proteins, with histone acetyl transferase (HAT) activity, are recruited (123).

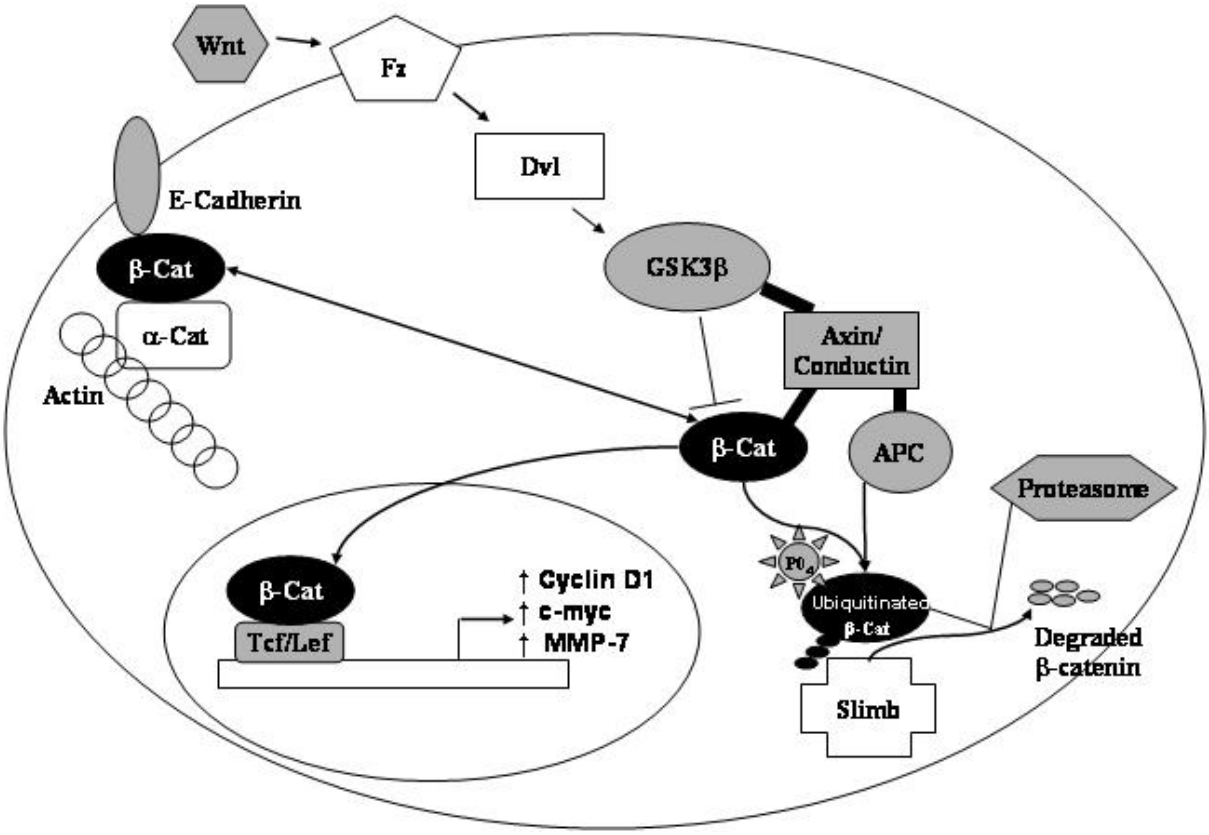

Figure 1.4 Schematic representation of Wnt signaling pathway. β-Cat, β-catenin; Fz, frizzled; Dvl, disheveled.

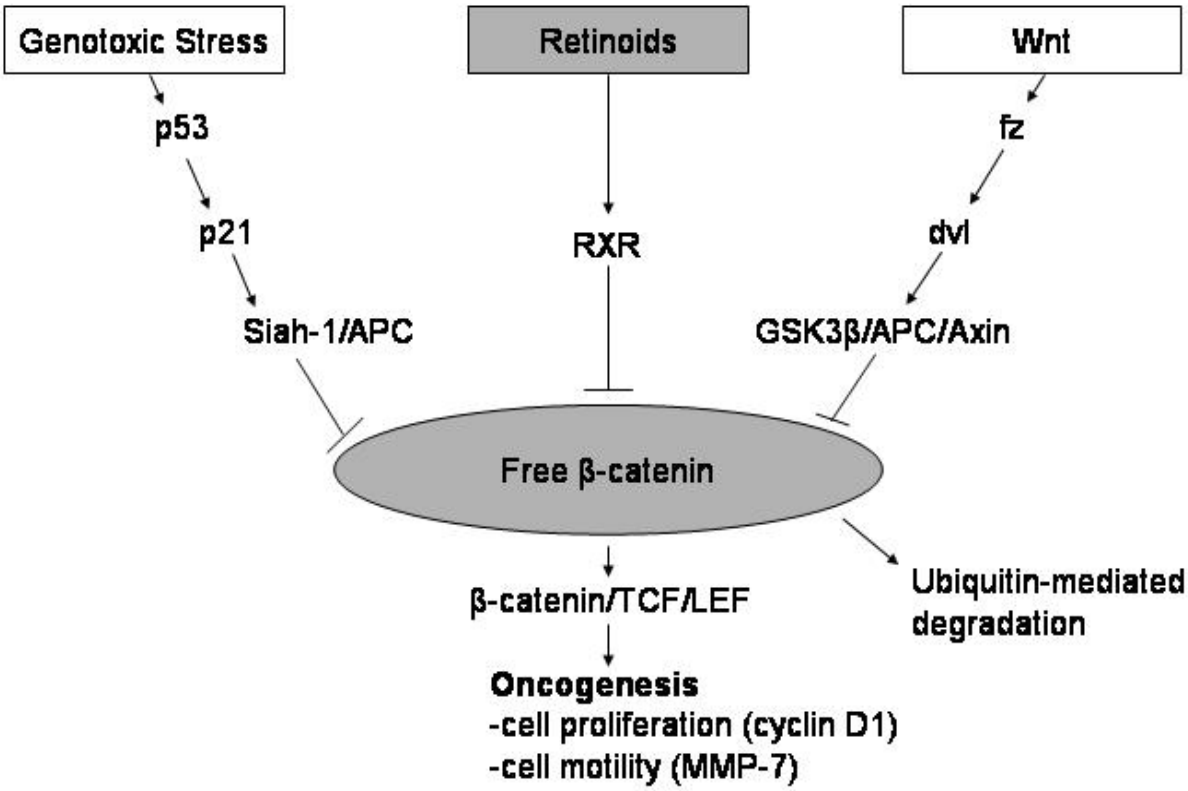

Figure 1.5 Schematic model of three β-catenin degradation pathways. Fz, frizzled; dvl, disheveled. Adapted from (96).

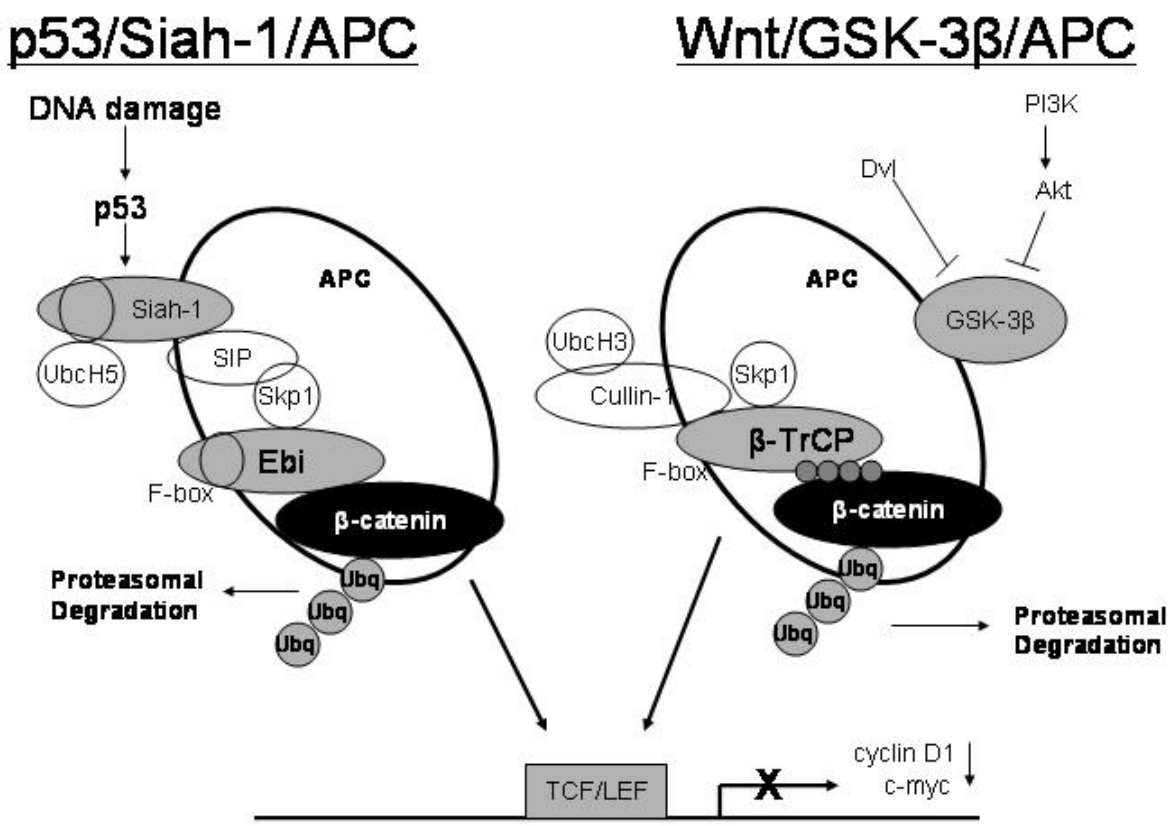

Figure 1.6 Model of Wnt/GSK-3β/APC and p53/Siah-1/APC pathways for β-catenin degradation. The p53 pathway involves protein interactions with SIP, Skp1, and Ebi proteins. Ebi binds β-catenin and the complex marks it with ubiquitin. The Wnt signaling pathway is dependent on β-catenin phosphorylation, where β-TrCP can bind and then the complex can mark β-catenin with ubiquitin. APC is required for both pathways as a scaffolding protein, binding β-catenin via Siah-1 or GSK-3β (95).

Chapter 2: Retinol Inhibits the Growth of *All-Trans*-Retinoic Acid-Sensitive and -Resistant Colon Cancer Cells through a Retinoic Acid Receptor-Independent Mechanism

ABSTRACT

Retinol (vitamin A) is thought to exert its effects through the actions of its metabolite, *all-trans*-retinoic acid (ATRA), on gene transcription mediated by retinoic acid receptors (RAR) and retinoic acid response elements (RARE). However, RA-resistance limits the chemotherapeutic potential of ATRA. We examined the ability of retinol to inhibit the growth of ATRA-sensitive (HCT-15) and ATRA-resistant (HCT-116, SW620, and WiDr) human colon cancer cell lines. Retinol inhibited cell growth in a dose-responsive manner. Retinol was not metabolized to ATRA or any bioactive retinoid in two of the cell lines examined. HCT-116 and WiDr cells did convert a small amount of retinol to ATRA, however this amount of ATRA was unable to inhibit cell growth. To show that retinol was not inducing RARE-mediated transcription, each cell line was transfected with pRARE-CAT (chloramphenicol acetyltransferase) and treated with ATRA and retinol. Although treatment with ATRA did increase CAT activity five-fold in ATRA-sensitive cells, retinol treatment did not increase CAT activity in any cell line examined. To demonstrate that growth inhibition due to retinol treatment was independent of ATRA, RAR, and RARE, a pan RAR-antagonist was used to block RAR-signaling. Retinol-induced growth inhibition was not alleviated by the RAR-antagonist in any cell line, but the antagonist did alleviate ATRA-induced growth inhibition of HCT-15 cells. Retinol did not induce apoptosis, differentiation or necrosis, but did affect cell cycle progression. Our data show that retinol acts through a novel, RAR-independent mechanism to inhibit colon cancer cell growth.

INTRODUCTION

Colorectal cancer is currently the third leading cause of death due to cancer in the United States. Retinoids, a group of compounds consisting of vitamin A (retinol), its natural metabolites, and several synthetic compounds, have been shown to act as cancer chemopreventive agents [for reviews see: (124-126)]. Retinol, *9-cis*-retinoic acid (*9-cis*-RA), and 4-(hydroxyphenyl)retinamide (4-HPR) can inhibit the formation of carcinogen-induced aberrant crypt foci, a precursor to colon cancer, as well as colon tumors in rats (34,38,40,41). Retinyl palmitate was recently shown to inhibit high fat diet-induced aberrant crypt foci (34). In addition, several *in vitro* studies illustrate that retinoids have potent antiproliferative effects on colon cancer cell lines and may hold potential for both chemoprevention and chemotherapy of colon cancer.

In almost all of the above studies, the retinoid examined has been an isoform of RA or a synthetic retinoid such as 4-HPR. Although these compounds are effective at inhibiting *all-trans*-RA (ATRA)-sensitive cell growth, the use of exogenous ATRA to study the effects vitamin A assumes that all of the biological phenomena attributed to retinol are due to ATRA. The diet contains very little ATRA (14). Rather, the diet contains vitamin A as: 1) previtamin A carotenoids and 2) preformed vitamin A as retinol and retinyl esters. Retinyl esters are cleaved within the intestinal lumen to yield retinol. Therefore, human colonocytes are exposed primarily to retinol, the focus of this study. Within most cells, retinol is either esterified for storage or metabolized to ATRA. ATRA effects cell growth and differentiation by binding to retinoic acid receptors (RARs), located in cell nuclei. RARs heterodimerize with retinoid X receptors (RXRs) and bind to retinoic acid response elements (RAREs) located in the regulatory regions of

retinoid-responsive genes. When ATRA binds to the RAR member of the RAR/RXR heterodimer, gene transcription via RARE is induced [for review see: (125)].

RA-resistance is believed to be due to a defect in RAR α, β, or γ induction in response to ATRA [for review see: (42,45,46,125)]. RA-resistance occurs when tumors or tumor-derived cell lines cease to growth inhibit or differentiate in response to treatment with ATRA. Retinoic acid resistance is a common phenomenon and appears to arise spontaneously in numerous types of cancer and tumor-derived cell lines. The defective receptor varies with cell line but RARβ expression is frequently lost.

The objective of the present study was to determine if retinol inhibits the growth of both ATRA-sensitive and ATRA-resistant colon cancer cell lines *in vitro*. Because the ATRA-resistant cell lines lack one or more RARs, their use allowed us to determine the effects of retinol on cell growth, exclusive of the effects of ATRA. Our data show that retinol itself inhibits the growth of both ATRA-sensitive and ATRA-resistant colon cancer cells through an ATRA and RAR independent mechanism.

MATERIALS AND METHODS

Tissue Culture

Three human colorectal adenoma cell lines, HCT-15, SW620, and WiDr, and one human colon carinoma cell line, HCT-116, were obtained from the American Type Culture Collection (Manassas, VA) and grown as recommended. HCT-15 cells were grown in MEM, HCT-116 in McCoy's medium, and SW620 and WiDr cells in DMEM in a humidified atmosphere at 37°C with 5% CO_2. All medium was supplemented with 10% FCS (fetal calf serum) and antibiotics (1000 U/mL penicillin and 1000 μg/mL streptomycin). The experiment was repeated with each cell line grown in either supplemented DMEM or McCoy's medium. Medium type did not affect

cell growth. Cells were seeded in 12-well culture dishes at a density of 1 x 10^4 cells per well. The following day the medium was removed and replaced with medium containing 0, 0.1, 1 or 10 μM ATRA or *all-trans*-retinol (Sigma, St Louis, MO). All retinoids were prepared as 10 mM stocks in 100 % ethanol. All treatments, including control, received equal volumes of ethanol vehicle and all retinoid manipulations were performed under subdued lighting. All treatments were performed in duplicate. Cells were harvested using trypsin and counted via hemocytometer every 24 h for four days.

Retinoid Extraction and HPLC Analysis.

To examine retinol metabolism, cells were seeded in 60 mm dishes at the following densities to yield 60 to 80% confluency and maximum HPLC detection sensitivity at the time of harvest: 5 x 10^5 cells/dish for 24 h, 2.5 x 10^5 cells/dish for 48 h, 1 x 10^5 cells/dish for 72 h, and 5 x 10^4 cells/dish for 96 h. Twenty-four hours after plating, cells were treated with 0, 1, and 10 μM retinol for 24, 48, 72 or 96 h. Sixteen hours before harvest, the culture medium was removed and replaced with medium containing 5% FCS and 50 nmol/L [^{3}H]retinol (specific activity = 52.5 Ci/mmol). Cells and medium were harvested 2, 4, 8, and 16 h after the addition of label as described previously (127). A control of labeling medium without cells was also incubated for 16 h. F9 murine teratocarcinoma cells, treated with 1 μM ATRA for 48 hr and incubated with 50 nM [^{3}H]-retinol for 16 hr were used as a positive control for 4-oxoretinol production (23). Retinoids were extracted and separated using a Waters Millennium HPLC system as described previously (24).

Cell Transfection and CAT Assays.

To examine the possibility that an undetected metabolite of retinol was activating RAR/RXR-mediated transcription, all cell lines were transiently transfected with pRARE-CAT

(generously provided by Dr. Dianne Soprano, Temple University, Philadelphia, PA). Cells were seeded on to 12-well plates at a density of 1.75 $X10^5$ cells/well, and incubated overnight in FCS-supplemented medium. The following day cells were transfected using Lipofectamine 2000 (Promega, Madison, WI) according to the manufacturer's protocol with 1 μg of pRARE-CAT and 0.5 μg of pSV-β-gal. Twenty-four hours later the transfection medium was removed and the cells were treated with fresh medium containing 0, 1, and 10 μM ATRA or retinol. The cells were harvested after treatment for 24 or 48 h and assayed for β-galactosidase (β-Galactosidase Enzyme Assay System, Promega, Madison, WI) and CAT (CAT Enzyme Assay System, Promega, Madison, WI) activity as per manufacturer's instructions. CAT activity was corrected for transfection efficiency using the β-galactosidase activity.

RAR Antagonist Assays.

To determine if retinol was inhibiting cell growth via RAR, the pan-RAR antagonist, AGN 193109 was used to block RAR function. The RAR pan-antagonist was synthesized by Allergan, Inc. (Irvine, CA). Cell lines were plated at a density of 1 x 10^4 in 12-well plates and allowed to attach overnight. The following day, HCT-15 cells were treated with 0 and 1 μM ATRA or retinol with and without 10 μM AGN 193109. HCT-116, SW620, and WiDr cells were treated with 0 and 1 μM retinol with and without 10 μM AGN 193109. Control cells received an equal volume of DMSO and ethanol vehicle. Cells were harvested after treatment for 48 h (HCT-15) or 96 h (HCT-116, SW620, and WiDr). All treatments were performed in duplicate. The pharmacological, 10 μM, concentrations of ATRA and retinol were not examined because 100 μM AGN 193109 was toxic to the cells.

Detection of Apoptosis.

Nuclear staining via DAPI (4, 6-diamidino-2-phenylindole) and flow cytometry analysis, described below, were used to determine if cell growth inhibition was due to apoptosis. For DAPI staining, cells were plated at 1 x 10^4 cells per well in 12-well plates before treatment with 0, 1, and 10 μM retinol. Cells incubated for 4 hr at 37ºC with 4 μg/mL camptothecin served as the positive control for apoptosis. Both adherent and floating cells were harvested every 24 h for 4 d. The cells were centrifuged and washed with PBS to remove all traces of media. Cells were then incubated with 2 μg/mL DAPI for 10 min at 37ºC before counting at 400X magnification with an Olympus upright fluorescence microscope. To obtain cell counts, at least three different locations on each slide were used. Two hundred cells were counted at each location yielding a minimum of 600 cells counted per slide. Cells with segmented nuclei were scored as apoptotic.

Cellular Differentiation.

Alkaline phosphatase activity was used to determine if retinol was inhibiting cell growth by inducing cellular differentiation. All cell lines were plated on 60 mm dishes at a density of 5 x 10^4 cells per plate. Twenty-four hours later, cells were treated with 0, 1 and 10 μM retinol or 2 mM sodium butyrate (positive control) for 96 h. Alkaline phosphatase activity was determined as described previously (128). Alkaline phosphatase activity was measured by the conversion of *p*-nitrophenyl phosphate (19.8 mM) to *p*-nitrophenol by 0.1 mL of cell lysate in 100 mM glycine buffer, containing 1 mM $MgCl_2$, pH 10. Alkaline phosphatase enzyme activity was corrected for lysate protein content and expressed as percent positive control.

Necrosis assays.

Trypan blue exclusion assays were performed to measure cell death. Briefly, an aliquot of the cells harvested for the growth curve assays was pelleted by centrifugation, resuspended in

0.5 mL HBSS, and incubated with an equal volume of 0.4% trypan blue solution (Sigma-Aldrich, St. Louis, MO) for five minutes at room temperature before counting with a hemocytometer. Blue cells were scored as necrotic.

Flow Cytometry Analysis.

To determine if growth inhibition was due to cell cycle arrest and to confirm the absence of apoptosis through lack of a sub-G_1 peak, cells were seeded on 60 mm dishes at a density of 3 x 10^5 (HCT15 and SW620) or 2 x 10^5 (HCT-116 and WiDr) cells per dish to provide 50-60% confluence at the time of harvest. To synchronize, cells were plated in serum-free medium for 24 h and treated with 1 (HCT-15) or 3 μg/mL (HCT-116, SW620, WiDr) aphidicholin for an additional 24 hr. The following day, the cells were washed with PBS and treated with fresh FCS-supplemented media containing 0, 1, and 10 μM retinol. Cells were harvested, fixed in 70% ethanol overnight and stained with 40 μg/mL propidium iodide as described previously (129). At least 10,000 cells were analyzed per sample using a FACSCalibur machine (Becton Dickinson, San Jose, CA). DNA content was determined using Modfit software version 3.0 (Verity Software House, Inc, Topsham, ME).

Data Analysis

Statistical analyses were performed using Excel (XP 2002; Microsoft). Two-tailed, paired student's t-tests were performed to determine differences between vehicle control and retinol-treated cells. Data are expressed as mean ± SEM, n=3. Differences were considered significant at $P < 0.05$.

RESULTS

Growth of ATRA-resistant colon cancer cells is inhibited by retinol.

The ability of retinol to inhibit cell growth was examined in three ATRA-resistant human colon cancer cell lines HCT-116 (56), SW620 (120), and WiDr (42). HCT-15, an ATRA-sensitive cell line, was chosen to serve as a positive control for the inhibitory effects of ATRA on colon cancer cell growth (130). Serum concentrations of retinol range from 0.5 to 2 μM (131). Therefore, 0.1 μM was selected to represent a sub-physiological, and 1 μM a physiological, concentration of retinol. The highest level, 10 μM retinol, was used as a pharmacological, but potentially therapeutically relevant, concentration. There is very little ATRA (4-14 nM) in the serum (132,133). ATRA levels were chosen to match the concentrations of retinol used and to reflect ATRA levels commonly found in the literature (42,45,46,134).

After 96 h of treatment, the growth of HCT-15 cells was inhibited by ATRA (Figure 2.1A), as expected. In addition, HCT-15 cell growth was also inhibited by retinol in a dose-responsive manner. Cells treated with 10 μM retinol exhibited the largest degree of inhibition to 36.7 ± 7.8 % of control. HCT-116 cell growth was inhibited slightly by 0.1 and 1 μM retinol and this decrease was not significant when compared to the same concentrations of ATRA (Figure 2.1B). However, HCT-116 cell growth was significantly inhibited by 10 μM retinol (37.5 ± 9.2 % of control) when compared to 10 μM ATRA (74.3 ± 4.7 % of control). SW620 and WiDr cell growth was significantly inhibited by treatment with 0.1 and 1 μM retinol for 96 h when compared to ATRA (Figure 2.1C and D) indicating that physiological levels of retinol can inhibit the growth of ATRA-resistant cells. At 10 μM concentrations, there was no significant difference in the ability of ATRA and retinol to inhibit SW620 and WiDr cell growth. The highest concentration of ATRA, 10 μM, inhibited cell growth slightly in all cell lines examined (Figure 2.1). These data show that retinol can inhibit the growth of both ATRA-sensitive and ATRA-resistant colon cancer cells.

Retinol is not metabolized to bioactive compounds.

To determine if retinol was metabolized to a bioactive compound, such as ATRA, anhydroretinol (AR), or 4-oxoretinol, reverse-phase HPLC was performed on all cell lines as described previously (24). To ensure that no transient bioactive retinoids were overlooked, retinol metabolism was examined after 24, 48, 72 and 96 h of treatment with 0, 1, and 10 μM retinol followed by 2, 4, 8, and 16 h of incubation with 50 nM [^{3}H]-retinol.

HCT-15 and SW620 cells produced no [^{3}H] -ATRA, 4-oxoretinol, or AR from [^{3}H]-retinol at any time point or treatment (Figure 2.2). The absence of [^{3}H]-4oxoretinol was also confirmed by northern blot analysis that failed to show CYP261A mRNA expression in any of the four cell lines examined (data not shown). HCT-116 and WiDr cells did synthesize a small amount of [^{3}H]-ATRA from [^{3}H]-retinol (Figure 2.2). However, both control and retinol-treated cells metabolized [^{3}H]-retinol to [^{3}H]-ATRA. The time point displayed in Figure 2.2 (48 h of retinol treatment followed 8 h of incubation with 50 nM [^{3}H]-retinol) showed the largest concentration of 50 nM [^{3}H]-ATRA synthesis by HCT-116 and WiDr cells of any time point examined. When corrected for cell number, HCT-116 cells treated with the vehicle control synthesized 0.15 nM [^{3}H]-ATRA/million cells from 50 nM [^{3}H]-retinol. Cells treated with 10 μM retinol synthesized 0.55 nM [^{3}H]-ATRA/million cells from 50 nM [^{3}H]-retinol. Because the metabolism of 50 nM [^{3}H]-retinol reflects the metabolism of 10 μM retinol (24,127,135), we can assume that if HCT-116 cells were treated with 10 μM retinol, 0.11 μM of ATRA would be produced per million cells. There were 5.4 x 10^6 cells on a duplicate plate of HCT-116 cells treated with 10 μM retinol for 96 h and included in the experiment shown in Figure 2.2. As can be seen in Figure 2.1B, HCT-116 cell growth is inhibited only slightly by 0.1 or 1 μM ATRA when compared to control. The amount or [^{3}H]-ATRA synthesized from [^{3}H]-retinol by WiDr cells was even less then that synthesized by the HCT-116 cell line. Therefore, the small amount of ATRA

produced by these cell lines when treated with 10 μM retinol cannot be responsible for the decrease in cell number that occurs when these cells are treated with 10 μM retinol.

Retinol does not induce RARE-CAT reporter gene expression.

Each colon cancer cell line was transiently transfected with pRARE-CAT and treated with ATRA or retinol to confirm that retinol was not metabolized to a bioactive compound that could transactivate RARE-mediated gene transcription. The pRARE-CAT construct contains only the nucleotides corresponding to the RARE found in the regulatory region of the RARβ2 gene linked to a CAT promoter.

ATRA-sensitive HCT-15 cells show that treatment of with both 1 and 10 μM ATRA for 48 h resulted in an increase in CAT activity to 4.98 ± 0.39 -fold over control at 48 h (Figure 2.3A). Because HCT-15 cells were ATRA-sensitive we were surprised to find that treatment of HCT-15 cells with retinol did not increase CAT activity to more then 1.80 ± 0.06 -fold over control at 48 h for cells treated with 1 μM retinol (Figure 2.3A). However, this lack of CAT activity reflects the metabolism data (Figure 2.2A-D) showing that HCT-15 cells do not metabolize retinol to ATRA.

Neither ATRA nor retinol increased CAT activity more then 1.7 -fold over control in any of the three ATRA-resistant colon cancer cell lines (Figure 2.3B-D). The lack of CAT activity in cells treated with ATRA confirms the inability of these cells to respond to ATRA via RAR/RARE mediated mechanisms as described previously (42,56,120). Although the HCT-116 cells converted a small amount of [^{3}H]retinol to [^{3}H]ATRA, these cells lack RAR (56). The absence of an increase in CAT activity in response to retinol treatment in the HCT-116 and WiDr cell lines shows that the small amount of ATRA produced by these cells does not induce RAR/RARE-mediated gene transcription. SW620 cells did not metabolize [^{3}H]retinol to

[^{3}H]ATRA (Figure 2) and the lack of CAT activity in SW620 cells when treated with retinol both confirms the metabolism data and shows that an RAR-activating metabolite of retinol is either not present or is incapable of activating RAR/RARE-mediated gene transcription. In summary, the inability of retinol to increase CAT activity in any of the cell lines examined, including ATRA-sensitive HCT-15 cells, demonstrates that retinol is not inducing RA-mediated gene transcription, confirming our metabolism data, and indicating that retinol may be acting exclusive of the RAR to inhibit colon cancer cell growth.

Retinol is not acting through the RAR to inhibit cell growth.

To confirm that the growth inhibition exhibited by cells treated with retinol was not mediated by the RA/RAR/RARE retinoid signaling mechanism, all cell lines were treated with a RAR pan-antagonist, AGN 193109. This antagonist, when added at 10-times the concentration of agonist, blocks the ability of agonist to bind to RAR (136). HCT-15 cells treated with 1 μM ATRA and 10 μM AGN 193109 served as a positive control for the ability of AGN 193109 to block RAR-mediated cell growth inhibition. Because 1 μM ATRA does not inhibit HCT-116, SW620, or WiDr cell growth we did not test the effects of the combined treatment of AGN 193109 and ATRA in these cell lines. As shown in Figure 2.4A, AGN 193109 blocked ATRA-induced growth inhibition in HCT-15 cells, as expected. However, AGN 193109 did not block growth inhibition due to retinol treatment in any of the four cell lines examined, including the ATRA-sensitive HCT-15 cell line (Figure 2.4). The inability of AGN 193109 to block retinol-induced growth inhibition confirms the results of the metabolism and RARE-reporter experiments which also indicate that retinol is not acting via RA/RAR/RARE to affect cell growth even in the ATRA-sensitive, HCT-15 cell line. Unlike the ATRA-resistant cell lines, HCT-15 cells contain all of the cellular machinery required for induction of ATRA/RAR/RARE-

mediated gene transcription and growth inhibition (42). As shown in Figure 2.4A, ATRA is acting via this mechanism to inhibit the growth of HCT-15 cells. In contrast, retinol is acting via a novel, receptor-independent mechanism to inhibit the growth of both ATRA-resistant and surprisingly, ATRA-sensitive human colon cancer cell lines.

Retinol does not induce apoptosis, differentiation, or necrosis in ATRA-resistant colon cancer cells.

To determine the mode by which retinol inhibits the growth of colon cancer cells, apoptosis was examined by nuclear staining using DAPI (Figure 2.5, left column) and FACS analysis of DNA content (Figure 2.6). The percentage of DAPI-stained cells exhibiting segmented nuclei was less then 10% in all cell lines at all time points and treatments examined. FACS analysis failed to detect a sub-G_1 peak in any of the cell lines when treated with retinol, confirming the absence of apoptosis (Figure 2.6). Additionaly, in all cell lines, less than 4% apoptosis was detected with TUNEL assay and no apoptosis was detected by PAR-P cleavage or DNA laddering (data not shown). Therefore, retinol does not inhibit colon cancer cell growth by inducing apoptosis.

Alkaline phosphatase assays were performed to determine if retinol was inhibiting colon cancer cell growth by inducing cellular differentiation. Retinol does not induce alkaline phosphatase activity in HCT-116, SW620, or WiDr cells (Figure 2.5, right column). Retinol increased alkaline phosphatase activity slightly in HCT-15 cells (Figure 2.5A). In contrast, treatment with sodium butyrate resulted in a large increase in alkaline phosphatase activity in each cell line. These data indicate that retinol is not inhibiting cell growth by inducing cellular differentiation in the three ATRA-resistant cell lines. A small increase in alkaline phosphatase

activity in HCT-15 cells treated with retinol may indicate that cellular differentiation accounts for part of the retinol-induced decrease in growth.

To ensure that retinol was not inducing necrosis, trypan blue dye exclusion assays were performed on the adherent cells used for the growth curve experiments described in Figure 2.1. The percent of cells that stained with trypan blue dye varied between 0.1 and 7% and no consistent pattern was exhibited under any treatment condition at any time point (data not shown). Therefore, necrosis is not responsible for the growth inhibition exhibited by colon cancer cells treated with retinol.

Retinol affects cell cycle progression.

Treatment with 10 μM retinol increased the percentage of cells in $G_{0/1}$ while decreasing the percentage of cells in S-phase in the HCT-15, SW620, and WiDr cell lines (Figure 2.6 A, C, and D). Treatment with retinol decreased the percentage of HCT-15 cells in $G_{2/M}$, slightly increased the percentage of HCT-116 and SW620 cells in $G_{2/M}$, and notably increased the percentage of WiDr cells in $G_{2/M}$. In contrast, the percentage of HCT-116 cells in $G_{0/1}$ was not affected by retinol (Figure 2.6B), but retinol did decrease the percentage of HCT-116 cells in S-phase. As can be seen in Figure 2.1D, control HCT-116 cells continued to divide in a linear manner, while HCT-116 cells treated with 10 μM retinol ceased to divide between 24 and 48 h of treatment. This result, when considered in light of the absence of apoptosis, differentiation, and necrosis in the HCT-116 cell line despite strong growth inhibition by retinol, may indicate that retinol acts to slow the overall rate of cell division and increase the generation time of this cell line.

DISCUSSION

This study shows that retinol inhibits the growth of both ATRA-sensitive and ATRA-resistant human colon cancer cell lines. We provide three lines of evidence that retinol is acting independent of the established ATRA/RAR/RARE retinoid signaling pathway. The first line of evidence indicates that retinol is not metabolized to bioactive compounds, such as ATRA, in two out of the four cell lines examined. The remaining two cell lines synthesized only small amounts of ATRA from retinol. Second, we show that retinol does not activate RARE-mediated gene transcription. Finally, we present evidence that a RAR-antagonist blocks the ability of ATRA to inhibit the growth of ATRA-sensitive HCT-15 cells, as expected, but does not block the ability of retinol to inhibit the growth of any cell line examined. The most surprising outcome of this study is that retinol is not acting through a RAR-dependent pathway in ATRA-sensitive HCT-15 cells. Therefore, even in the presence of functioning RAR, retinol does not inhibit cell growth by the actions of its metabolite ATRA, because this metabolite is not present in ATRA-sensitive HCT-15 cells (Figure 2.2).

The ability of retinol to inhibit colon cancer cell growth is particularly interesting given that colon cancer cell lines produce little or no ATRA (Figure 2.2). This finding is supported in a recent study by Jette et al (137) that used northern blot analysis to show that colon cancer cell lines, including HCT-116, lack retinol dehydrogenases, and therefore the ability to synthesize ATRA. The metabolism of retinol by colon cancer cells was not examined in the study by Jette et al (137). In contrast, our data shows that the HCT-116 cell line is capable of synthesizing very small amounts of ATRA from retinol (Figure 2.2). This discrepancy is perhaps due to the ability of HPLC to detect extremely small amounts of ^{3}[H]-retinoids, compared to the relative lack of sensitivity of northern blot analysis.

4-Oxoretinol and AR are two naturally occurring retinoids capable of inhibiting cell growth. 4-Oxoretinol acts via RARs (23), much like ATRA, whereas AR acts via a receptor-independent cytosolic mechanism to inhibit cell growth (138,139). Neither compound was formed from retinol by any of the cell lines we examined (Figure 2.2). We cannot eliminate the possibility that an unknown bioactive metabolite of retinol was formed that existed only briefly or was not detected by our HPLC protocol. However, the metabolism data is supported by the inability of retinol to induce CAT-activity in cells transfected with a pRARE-CAT construct (Figure 2.3) as well as the inability of a pan-RAR antagonist to block the effects of retinol on cell growth (Figure 2.4).

We chose to use the pan-RAR antagonist, AGN 193109 to block RARs because this compound exhibits a high affinity for RAR (136). Although a genetic approach would have been more specific, the dominant negative RAR construct available is activated by retinol (140), making it inappropriate for this study. Therefore, we included a positive control, showing that AGN 193109 blocks ATRA-induced growth inhibition in the HCT-15, ATRA-sensitive cell line (Figure 2.3A), to indicate that AGN 193109 is functioning to block RAR-mediated growth inhibition.

Retinoids have been previously shown to inhibit cancer cell growth by increasing cellular differentiation, inducing apoptosis, or causing cell cycle arrest. With respect to colon cancer, retinoids tend to induce tumor apoptosis both *in vitro* (42,49,141) and *in vivo* (41,142). The cell lines examined in this study showed no apoptosis in response to retinol treatment (Figure 2.5 left column, Figure 2.6). In contrast, retinol induced $G_{0/1}$ arrest in three of the cell lines examined (Figure 2.6). Although retinol failed to increase the percentage of cells in $G_{0/1}$ in the HCT-116 cell line, growth inhibition in these cells could be due to an overall increase in generation time

because retinol does decrease cell growth (Figure 2.1B). The differing responses between the HCT-116 cell line and the other three may reflect the heterogenicity of these cell lines, tumor stage (carcinoma versus adenoma), and presence or absence of various proteins in each cell line, for example APC (adenomatous polyposis coli) or p53.

Retinoids tend to induce cell cycle arrest by blocking the G_1 to S phase transition [for review see: (126)]. Unfortunately, the effect of retinoids on cell cycle regulatory proteins appears to be cell type specific (126). For example, in carcinogen-exposed immortalized human bronchial epithelial cells, ATRA-induced G_1 arrest is associated with decreased cyclin D1 protein levels due to ubiquitin-mediated degradation of cyclin D1 (143,144). In contrast, ATRA-induced G_1 arrest in MCF-7 breast cancer cells is associated with decreased pRB phosphorylation, while cyclin D1, $p21^{WAF1/CIP1}$, cdk4 and cdk6 activity either does not change or decreases slightly, depending on the study (145-147).

This study shows that retinol is acting independent of the RAR to inhibit colon cancer cell growth. Previously, retinoids have been shown to exert their receptor-independent effects via interactions with protein kinase C alpha (PKCα) (148), F-actin (149), c-Raf kinase (138), regulating mitochondrial membrane potential (150), generating reactive oxygen species (151), increasing intracellular ceramide levels (152), activating c-Jun N-terminal kinase (153), inducing ubiquitin-dependent proteolysis (143,144), and affecting MAP kinase (154,155), phosphatidylinositol 3-Kinase (PI3K)/Akt (156), and epidermal growth factor receptor (EGFR) signaling (157). The Hammerling lab has shown that the retroretinoids, retinol, and ATRA can bind PKCα and affect its redox activation (148). In contrast to our present study, they speculate that retinol antagonizes AR and increases cell survival by binding to c-Raf and augmenting its response to reactive oxygen species generated during UV irradiation, however the link between

cell growth and c-Raf activation was not directly examined (138). Because AR induces apoptosis we do not expect retinol to be affecting cell growth by interacting with c-Raf or any of the other pathways listed above that induce apoptosis.

In conclusion, this study shows that retinol acts through a novel mechanism to inhibit the growth of both ATRA-sensitive and ATRA-resistant colon cancer cells by affecting cell cycle progression. Resistance to ATRA is a common phenomenon and limits the use of RA-derivatives as chemotherapy. We speculate that retinol, or a derivative of it, may prove an effective therapy to treat colorectal cancer.

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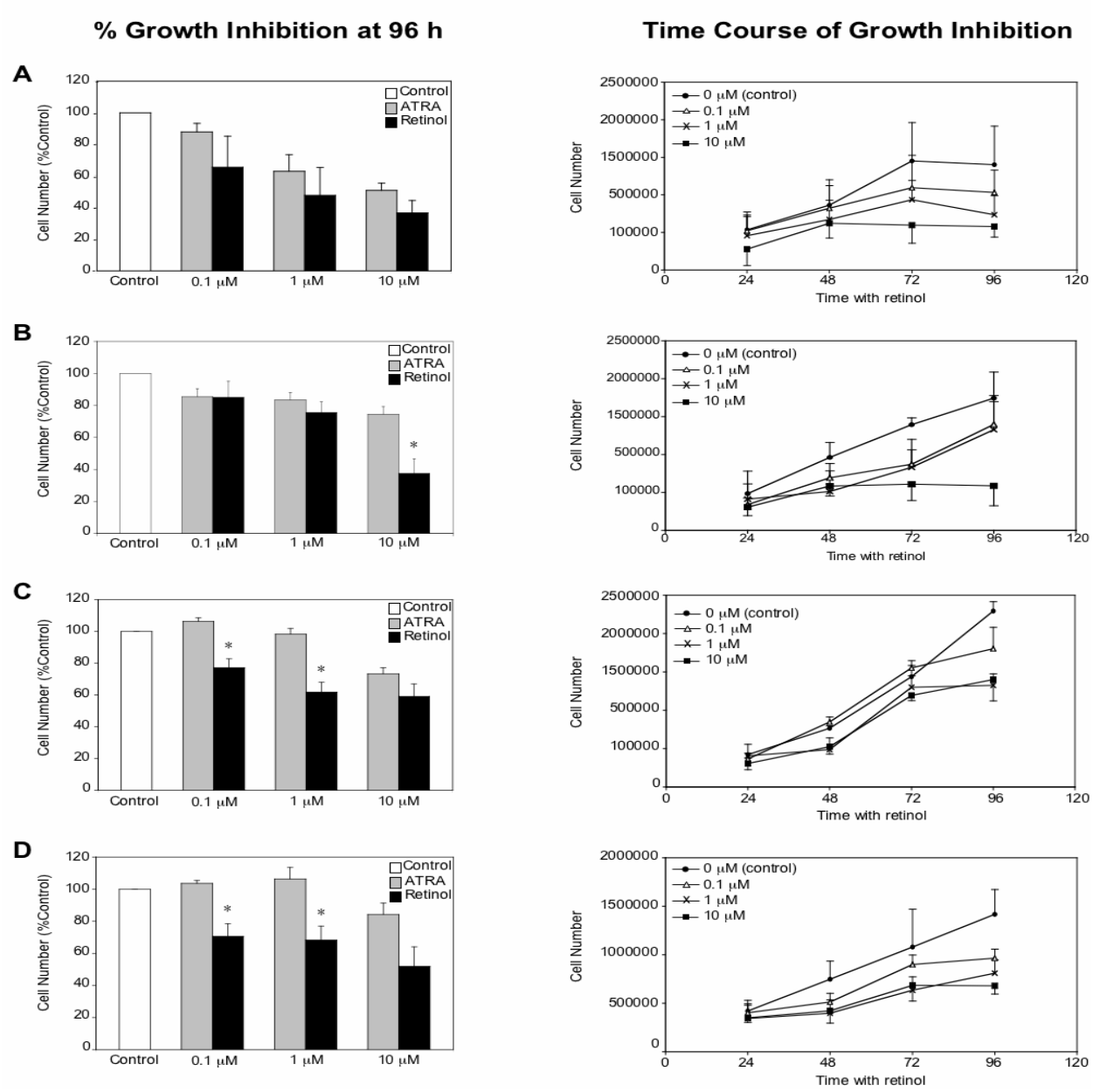

Figure 2.1 Retinol inhibits the growth of ATRA-resistant human colon cancer cells. HCT-15 (A) HCT-116 (B), SW620 (C), and WiDr (D) cells were seeded and treated with 0, 0.1, 1 or 10 μM ATRA or retinol. All treatments were performed in duplicate. Cells were counted via hemocytometer daily for four days. Results represent the mean ± SE for three experiments. Panels in the left column display the percent growth inhibition exhibited by human colon cancer cell lines after 96 h of treatment with increasing amounts of ATRA or retinol. Statistical analysis was performed using t-tests comparing ATRA to retinol for each concentration. *Indicates significantly different from ATRA, $P < 0.05$. Panels in the right column show the growth rates of HCT-15 (A), HCT-116 (B), SW620 (C) and WiDr (D) cells grown for four days with increasing amounts of retinol.

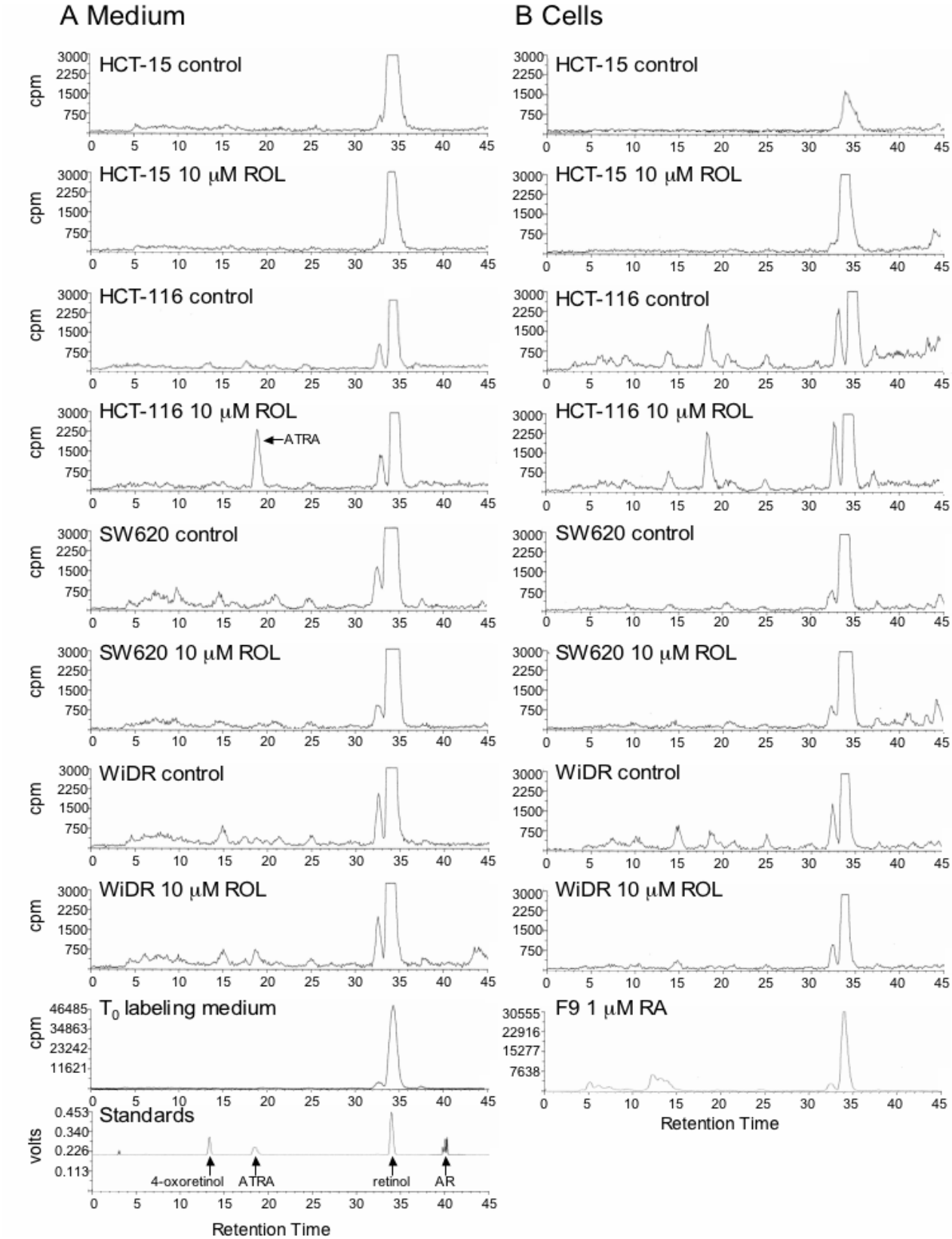
A Medium
B Cells
HCT-15 control
HCT-15 10 μM ROL
HCT-116 control
HCT-116 10 μM ROL
ATRA
SW620 control
SW620 10 μM ROL
WiDR control
WiDR 10 μM ROL
T0 labeling medium
F9 1 μM RA
Standards
4-oxoretinol
ATRA
retinol
AR
cpm
volts
Retention Time

Figure 2.2 Retinol is not metabolized to bioactive compounds in HCT-15 and SW620 cells but is converted to ATRA by HCT-116 and WiDr cells. Cells were plated and allowed to attach for 24 hr before addition of medium containing 0, 1, or 10 μM retinol. Cells were allowed to grow for 24, 48, 72 or 96 hr before the medium was removed and replaced with new medium containing 5% FCS and 50 nM [^{3}H]-retinol. The concentration of nonradioactive retinol in 5% FCS was ≈ 50 nM (24). Cells were allowed to incubate for another 2, 4, 8 or 16 hr before harvest. The retinoids were extracted from cell and medium samples and separated by HPLC as described (24). Data shown represent radiolabeled retinoids extracted from medium (A) or cells (B) of the indicated cell lines 48 h after treatment with 0 or 10 μmol/L retinol followed by incubation for 8 h with 50 nM [^{3}H]-retinol. The identities of the retinoids were determined by coelution with known nonradiolabeled (cold) standards included in the samples. Changes in absorbance are recorded as changes in voltage by the FloOne software that generated these chromatographs and controls the liquid scintillation counter. Thus, the units for the y-axis of "Standards" panel are volts. The slight difference in elution time between the cold standards and the [^{3}H]-peaks is due to the transit time from the photo diode array to the scintillation counter. [^{3}H]-Retinol extracted from labeling medium at time zero is shown in the left column, one panel from the bottom. F9 murine teratocarcimona cells were treated with 1 μmol/L RA for 48 hr followed by incubation with [^{3}H]-retinol for 16 hr prior to retinoid extraction. The retinoids extracted from F9 murine teratocarcimona cells are included as a positive control for 4-oxoretinol production and are shown in the last panel of the right column. Retinoid standards are shown in the bottom panel of the left column and eluted as follows: 4-oxoretinol, 13.5 min; ATRA, 18.5 min; all-*trans* retinol, 34.0 min, and AR, 39.8 to 40.5 min. This experiment was repeated twice with similar results.

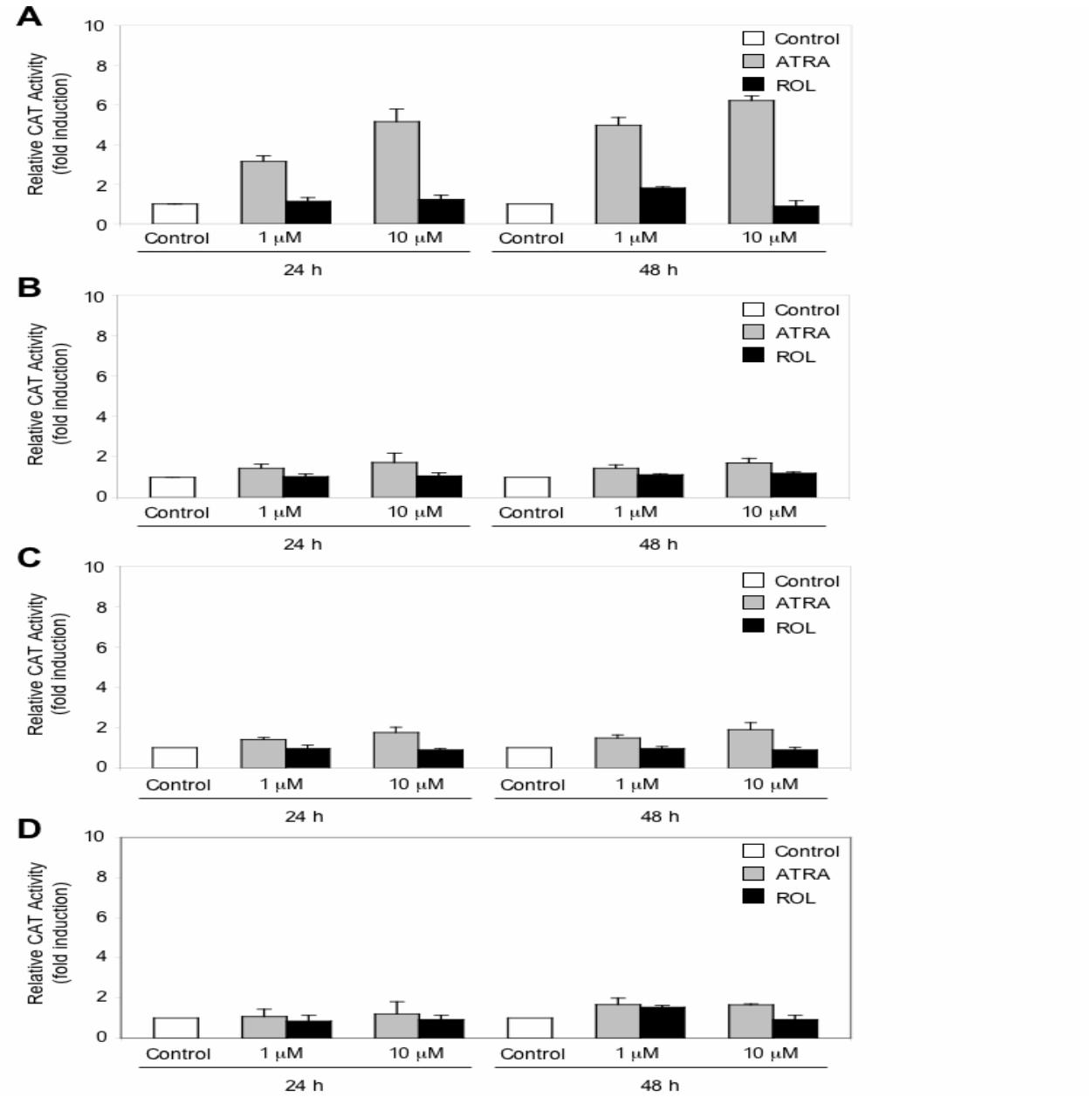

Figure 2.3 Retinol does not induce RARE-CAT reporter gene expression. HCT-15 (A), HCT-116 (B), SW620 (C), and WiDr (D) cells were transiently transfected with 1 μg pRARE-CAT and 0.5 μg pSV-β-gal using lipofectamine 2000. Twenty-four hours following transfection, cells were treated with 0, 1, and 10 μM RA or retinol. Cells were harvested 24 and 48 h after treatment and CAT and β-galactosidase assays were performed. CAT activity was normalized for transfection efficiency using the β-galactosidase activity. The CAT activity in control cells treated with the ethanol vehicle was set equal to one and all other values are expressed as fold induction. Values shown are the mean ± SE of three separate experiments.

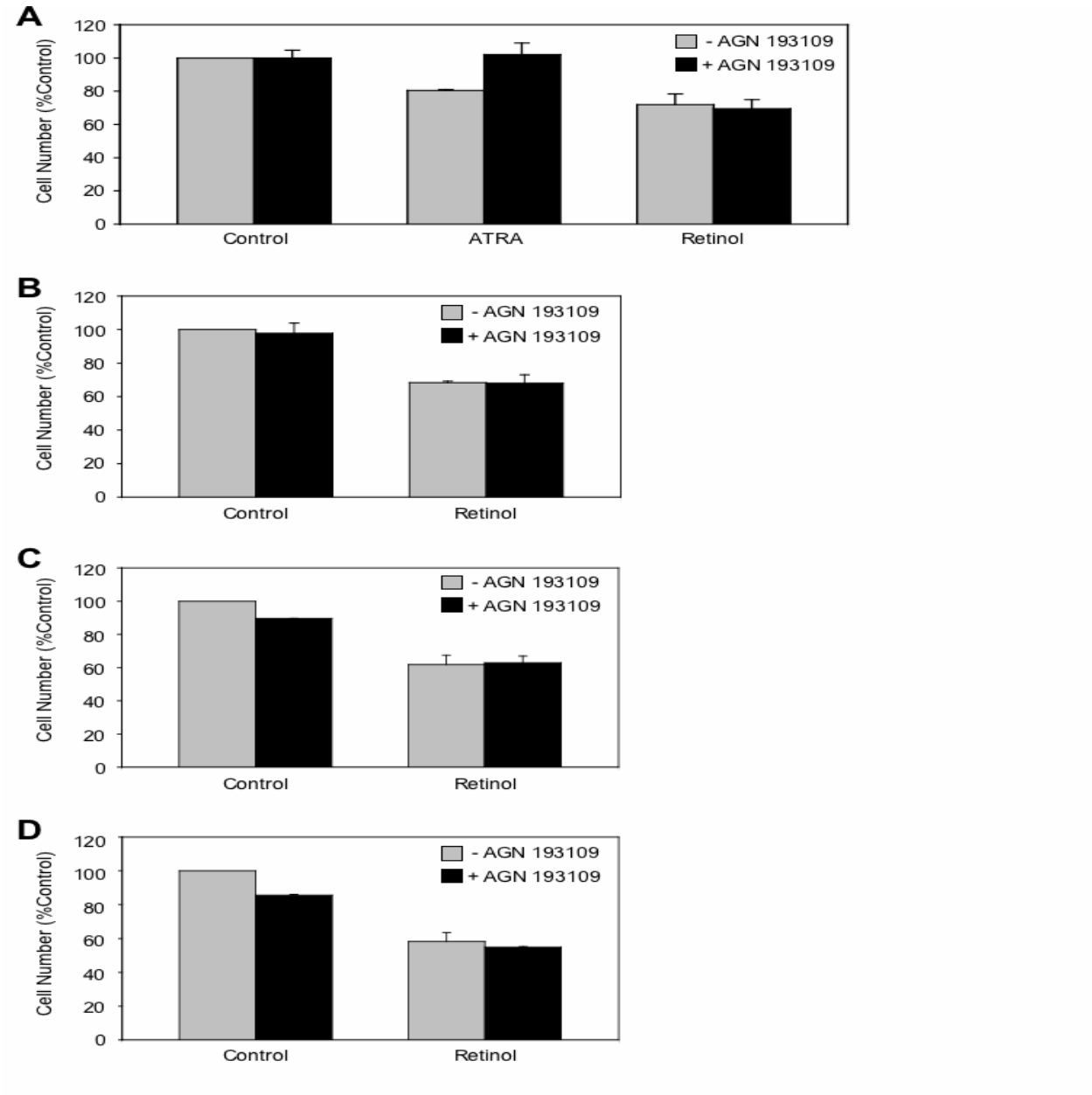

Figure 2.4 Retinol does not act through RAR to inhibit cell growth. HCT-15 (A) cells were plated with 0 or 1 μM RA or retinol with or without 10 μmol/L of the RAR pan-antagonist, AGN 193109, for 48 h. HCT-116 (B), SW620 (C), and WiDr (D) were treated with 0 or 1 μM retinol with or without 10 μM AGN 193109 for 96 h. All treatments were plated in duplicate. Data shown are the mean of two separate experiments ± SE.

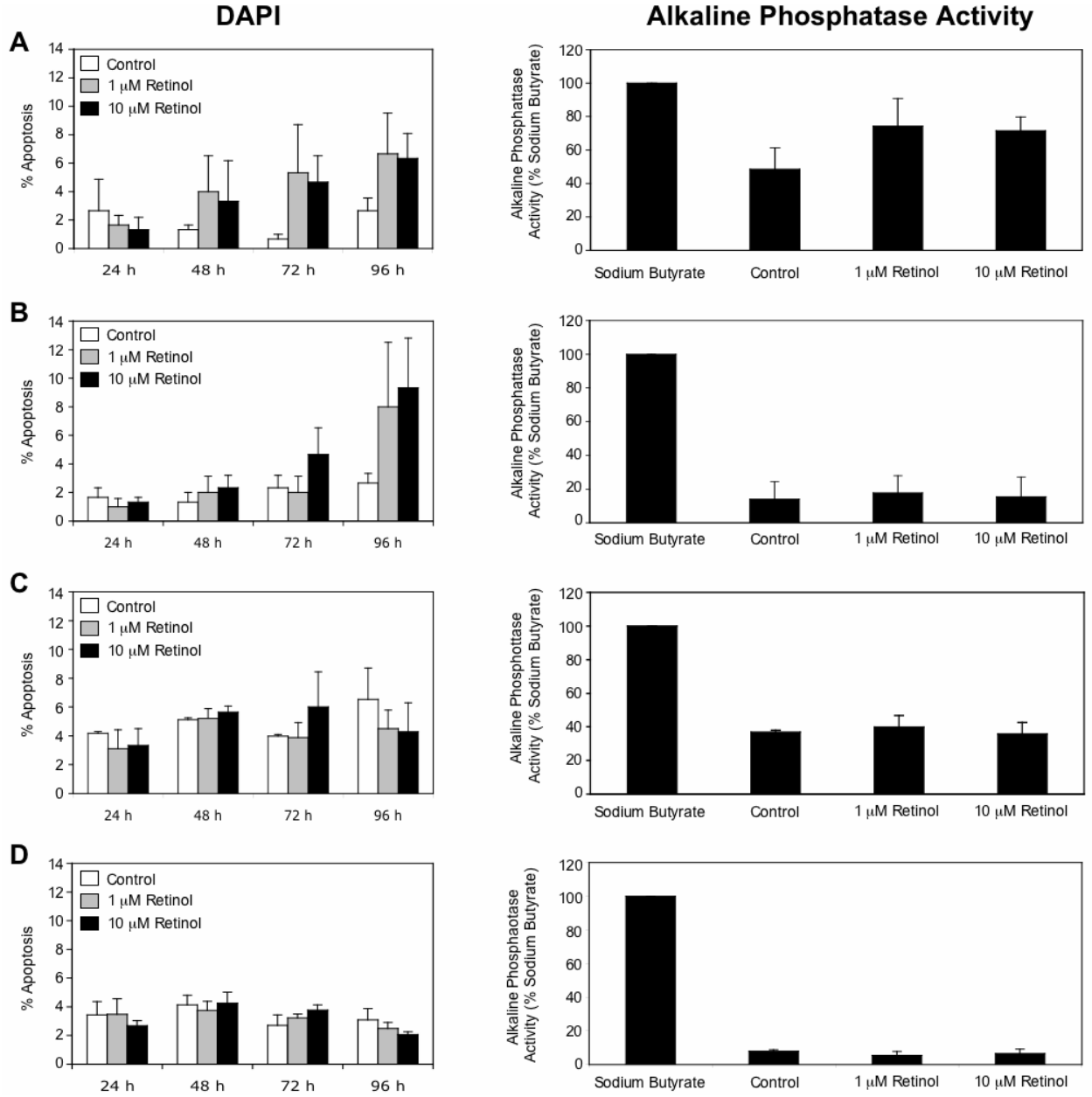

Figure 2.5 Retinol does not induce apoptosis or cellular differentiation in ATRA-resistant human colon cancer cell lines. HCT-15 (A), HCT-116 (B), SW620 (C), and WiDr (D) cells were plated and treated with 0, 1 or 10 μM retinol as described in Materials and Methods. To measure apoptosis (left column), floating and adherent cells were harvested every 24 hr, centrifuged and washed with PBS to remove all traces of media. Cells were stained with 2 μg/mL DAPI for 10 min in 37ºC before observation. To measure cellular differentiation (right column), cells were plated as described and treated with 0, 1, or 10 μM retinol or 2 mM sodium butyrate for 96 h. Alkaline phosphatase activity was determined as described (128) by the conversion of *p*-nitrophenyl phosphate to *p*-nitrophenol at 410 nm. Alkaline phosphatase enzyme activity is expressed as percent positive control. Data shown are the mean ± SE for three independent experiments.

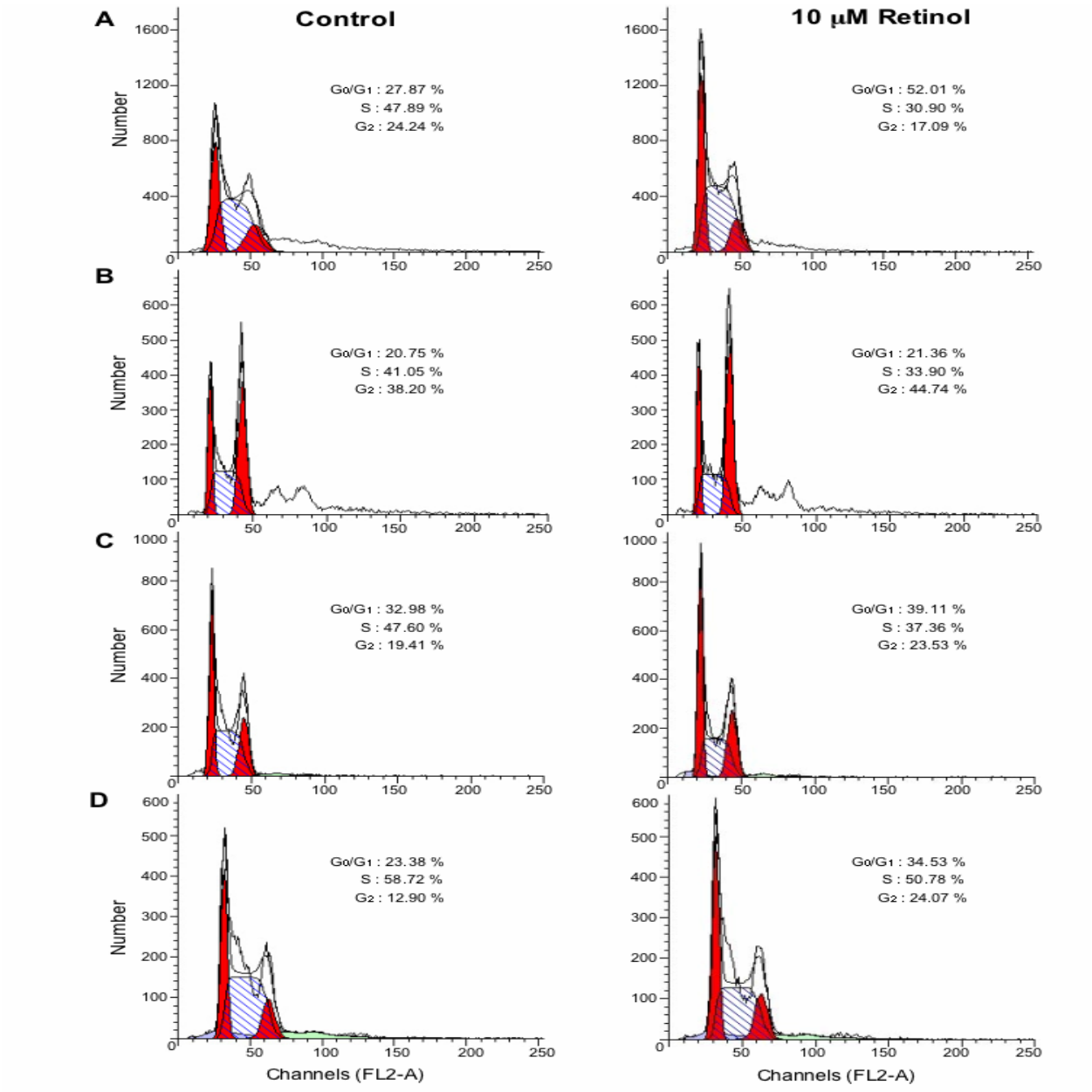

Figure 2.6 Retinol alters cell cycle progression but does not induce apoptosis. HCT-15 (A), HCT-116 (B), SW620 (C), and WiDr (D) cells were synchronized with serum starvation for a total of 48 h and the addition of aphidicholin during the last 24 h of serum starvation. Following synchronization, fresh medium containing 10 % FCS and 0 (left column) or 10 µM retinol (right column) was added for 24 h, prior to fixation and staining with propidium iodide. At least 10,000 cells were analyzed per sample using a FACS Calibur machine. Absence of a sub-$G_{0/1}$ indicates that apoptosis was not induced by retinol treatment. Dark gray, $G_{0/1}$ and $G_{2/M}$; hatched, S-phase. One representative experiment of two is shown.

Chapter 3: Retinol Decreases β-Catenin Protein Levels in Retinoic Acid-Resistant Colon Cancer Cell Lines

ABSTRACT

The β-catenin signaling pathway is dysregulated in most cases of colon cancer resulting in an accumulation of nuclear β-catenin and increased transcription of genes involved in tumor progression. This study examines the effect of retinol on β-catenin protein levels in three *all-trans* retinoic acid (ATRA)-resistant human colon cancer cell lines: HCT-116, WiDr, and SW620. Each cell line was treated with increasing concentrations of retinol for 24 or 48 h. Retinol reduced β-catenin protein levels and increased ubiquitinated β-catenin in all cell lines. Treatment with the proteasomal inhibitor, MG132, blocked the retinol-induced decrease in β-catenin indicating retinol decreases β-catenin by increasing proteasomal degradation. Multiple pathways direct β-catenin to the proteasome for degradation including a p53/Siah-1/adenomatous polyposis coli (APC), a Wnt/glycogen synthase kinase-3β/APC, and a retinoid "X" receptor (RXR)-mediated pathway. Due to mutations in β-catenin (HCT-116), APC (SW620), and p53 (WiDr) only the RXR-mediated pathway remains functional in each cell line. To determine if RXRs facilitate β-catenin degradation, cells were treated with the RXR pan-antagonist, PA452, or transfected with RXRα siRNA. The RXR pan-antagonist and RXRα siRNA reduced the ability of retinol to decrease β-catenin protein levels. Nuclear β-catenin induces gene transcription via interaction with TCF/LEF (T cell factor/lymphoid enhancer factor) proteins. Retinol treatment decreased the transcription of a TOPFlash reporter construct and mRNA levels of the endogenous β-catenin target genes, cyclin D1 and c-myc. These results indicate that retinol may reduce colon cancer cell growth by increasing the proteasomal degradation of β-catenin via a mechanism potentially involving RXR.

INTRODUCTION

The retinoids, a family of compounds consisting of vitamin A (retinol) and its derivatives, exhibit potent inhibitory effects on tumor cell growth [for reviews see: (14,30,125)]. Preformed vitamin A is primarily ingested as retinyl esters which are hydrolyzed to yield retinol in the intestinal lumen. The diet contains very low amounts of *all-trans* retinoic acid (ATRA) (14). Within the cell, retinol can be esterified and stored as retinyl esters or converted to ATRA. ATRA binds to the nuclear retinoic acid receptors (RARs), which function as heterodimers with retinoid "X" receptors (RXRs) to regulate the expression of target genes containing retinoic acid responsive elements (RAREs) in their promoter regions. However, as cancer progresses, cells frequently become resistant to the growth inhibitory effects of ATRA, usually due to silencing of the RAR genes or loss of the ability to induce the transcription of RARs in response to ATRA [For review see (30)]. The phenomenon of ATRA-resistance limits the effectiveness of ATRA as a cancer chemotherapy. Previous work in our laboratory has shown that retinol can inhibit the growth of ATRA-resistant human colon cancer cell lines by affecting cell cycle progression via a novel ATRA, RAR, and RARE-independent mechanism (158). In addition, dietary supplementation with the retinyl ester, retinyl palmitate, was recently shown to decrease the incidence of preneoplastic aberrant crypt foci in rats administered 1,2-dimethylhydrazine and consuming a high fat diet (34,159). Dietary retinyl palmitate also prevented the high fat diet-induced increase in colonocyte β-catenin protein levels (159).

β-Catenin is found in three locations within the cell: 1) the membrane-bound adherens complex, where it functions in cell to cell adhesion, 2) the nucleus, where it stimulates gene transcription, and 3) the cytoplasm where it serves as a pool for translocation to the membrane or nucleus or can be targeted for degradation. The degradation of cytosolic β-catenin is controlled

in one of three ways: 1) by the serine/threonine kinase, glycogen synthase kinase (GSK)-3β, which is part of the Wnt signaling pathway, 2) by the p53/Siah-1 pathway, and 3) by a nuclear hormone receptor-mediated degradation pathway. Mutations in the β-catenin degradation pathway are regarded as crucial for colon carcinogenesis and are present in 70-80% of colorectal tumors (84,89). These mutations hinder β-catenin degradation leading to increased nuclear β-catenin. Nuclear β-catenin induces gene expression through the T cell factor/lymphoid enhancer factor (TCF/LEF) transcription complex and modulates the transcription of genes involved in cell proliferation (e.g. cyclin D1 and c-myc) (77,78) and metastasis (e.g. matrix metalloproteinase-7) (79).

The effect of nuclear receptors on β-catenin protein levels and β-catenin/TCF/LEF-mediated gene transcription varies with nuclear receptor and cell types. In general, retinoid receptors either decrease β-catenin protein levels, as in the case of RXR, or inhibit β-catenin-mediated gene transcription, as in the case of RAR. For example, RXR decrease β-catenin mediated gene transcription by inducing the proteasomal degradation of β-catenin in several cell lines including APC-null and p53-mutant colon cancer cell lines (96). In contrast, SKBR3 breast cancer cells, which express low endogenous levels of β-catenin (114,115), exhibit increased β-catenin levels due to enhanced β-catenin protein stability when treated with 9-*cis*-retinoic acid (9-*cis*-RA), a ligand for both RAR and RXR (116). β-catenin levels were not affected by 9-*cis*-RA treatment in the MCF7 breast cancer cell line (105), but 9-*cis*-RA treatment did decrease β-catenin/TCF/LEF mediated gene transcription in MCF7, CaCo-2, HS578t and SKBR3 cells (105). An additional study by the same group showed that 9-*cis*-RA treatment reduced cytoplasmic levels of exogenously expressed β-catenin in SKBR3 cells by targeting β-catenin to the cell membrane (117). Also, the transcription of a cyclin D1 reporter construct lacking TCF

binding sites was decreased by 9-*cis*-RA in SKBR3 cells, indicating that AP-1 mediated cyclin D1 transcription in this cell line. In contrast to the SKBR3 cell line, 9-*cis*-RA treatment decreased the transcription of a wild type (wt) cyclin D1 reporter construct, containing TCF binding sites, in the SW480 colon cancer cell line, which expresses high levels of β-catenin. However, 9-*cis*-RA did not alter the transcription of a cyclin D1 reporter construct lacking TCF binding sites, indicating that β-catenin/TCF/LEF complex regulates cyclin D1 transcription in SW480 colon adenoma cells. The effects of 9-*cis*-RA on β-catenin/TCF/LEF-mediated gene transcription are due to the ability of ligand-bound RAR to compete with TCF for β-catenin binding. This competition prevents β-catenin-mediated gene transcription (105). It was later shown that the co-activator, p300, facilitates the interaction between RAR and β-catenin protein (160). These data illustrate that the effects of retinoids on β-catenin levels and signaling vary with cell line due to factors such as the amount of endogenous β-catenin and receptor compliment.

We previously showed that retinol decreases the proliferation of ATRA-resistant human colon cancer cells by slowing the progression from the $G_{0/1}$ to the S phase of the cell cycle (158), a process mediated by cyclin D1 transcription. The transcription of cyclin D1, in turn, is regulated by β-catenin in cells expressing high endogenous levels of β-catenin (117). The ATRA-resistant cell lines used in the previous and current study express high levels of β-catenin and lack some or all RARs (42,56,117,120,161) but express RXRs. Therefore, the objective of the present study was to determine the effect of retinol on total cellular β-catenin levels in ATRA-resistant human colon cancer cell lines. In the current study we show that retinol decreases total β-catenin protein levels in a dose-dependent manner in all three cell lines and that this reduction in β-catenin is due to an increase in ubiquitin-mediated proteasomal degradation.

This degradation occurs despite each cell line containing a mutation in one or more of the β-catenin degradation pathways, leaving the RXR-mediated β-catenin degradation pathway the functional pathway in common between all three cell lines. The use of a RXR antagonist and RXRα silencing small interfering RNA (siRNA) decreased the ability of retinol to induce the degradation of β-catenin indicating that RXRs may facilitate this process. Our data also show that retinol decreases β-catenin/TCF/LEF mediated gene transcription, leading to a decrease in mRNA levels of endogenous targets such as cyclin D1 and c-myc.

MATERIALS AND METHODS

Tissue Culture

The ATRA-resistant HCT-116, WiDr, and SW620 human colon cancer cell lines were obtained and cultured as recommended by the American Type Culture Collection (Manassas, VA). HCT-116, WiDr, and SW620 cells were cultured in McCoy's, MEM, and DMEM media, respectively, supplemented with 10% fetal bovine serum and antibiotics (1000 U/ml penicillin and 1000 μg/ml streptomycin). MEM media was also supplemented with 1 mM non-essential amino acids (Cellgro, Herndon, VA) and 10 mM sodium pyruvate (Cellgro, Herndon, VA). For all experiments, cells were plated in 60 mm culture dishes unless otherwise specified. The following day the medium was removed and replaced with media containing retinoids. All retinoids were prepared as 10 mM stock solutions in 100% ethanol. All treatments, including control, received equal volumes of the ethanol vehicle. All retinoid manipulations were performed under subdued lighting. Each experiment was repeated three times.

Western Immunoblot Analysis

To examine the effect of retinoids on β-catenin protein levels, HCT-116, WiDr, and SW620 cells were plated at a density of 1.25×10^5 cells per ml. The following day the medium

was removed and replaced with medium containing 0, 0.1, 1, and 10 μM ATRA or retinol. Cells were collected 24 and 48 h after treatment. Cells were lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, 1 mg/ml leupeptin, 1 mM DTT, 2 mM $NaOV_4$, 1 mg/ml PMSF, 1 mg/ml trypsin inhibitor, and 10 mM aprotinin) and quantified using the BioRad DC protein assay kit (Hercules, CA). Protein (25 μg) was electrophoresed through a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. Membranes were blocked with 5% milk in TBST (10 mM Tris, pH 8, 150 mM NaCl, and 0.5% Tween-20) for 1 h at room temperature before β-catenin polyclonal antibody (Transduction Laboratories, catalogue #610157, Beckton Dickinson, CA) and β-actin antibody (Sigma, catalogue #A2066, St. Louis, MO) were added at a 1:1000 dilution and a 1:5000 dilution, respectively. After incubation with the corresponding secondary antibody at a dilution of $1:1x10^4$, immunoreactivity was detected using the Pierce Horseradish Peroxidase Super Signal West Pico Chemiluminescent Substrate kit (Rockford, IL).

To determine if retinol increased the ubiquitination of β-catenin, all three cell lines were plated as specified above and treated with 0 and 10 μM retinol for 24 h. After protein collection, 50 μl of pre-washed protein A sepharose CL-4B beads (GE Healthcare Bio-Sciences AB, catalogue #17-0780-01, Sweden) and 500 μg of total protein lysate were incubated overnight with constant shaking at 4ºC. Because β-catenin is found in high amounts in all three ATRA-resistant colon cancer cell lines, this pre-wash was required to decrease background. Twenty-four hours later the supernatant was transferred to a tube containing β-catenin antibody at a 1:1,000 dilution. The protein and antibody were incubated together overnight with constant shaking at 4ºC. After 24 h, an additional 50 μl of pre-washed protein A sepharose beads were added to the protein/antibody mixture. This mixture was again incubated overnight with

constant shaking at 4ºC. After washing with RIPA three times, the bound β-catenin was eluted with 20 μl of 10 mM Tris-HCl, pH 6.8, 1% SDS at 55ºC for 5 min. The eluted samples were electrophoresed as described above. Membranes were blocked with 5% milk in TBST for 1 h, probed with the anti-ubiquitin antibody (BD Pharmigen, catalogue #550944, Beckton Dickinson, CA) at a 1:1000 dilution in TBST for 1 h. After incubation with the corresponding secondary at a dilution of $1:1x10^4$, immunoreactivity was detected as stated above. Membranes were then striped and probed for β-catenin.

To determine if retinol decreased β-catenin by increasing proteasomal degradation, all three cell lines were plated as specified above and treated with 0, 1, and 10 μM retinol with and without 1 μM MG132 (BIOMOL Research Labs, Plymouth Meeting, PA) for 24 h. To examine the effect of a RXR pan-antagonist on β-catenin protein levels, all three cell lines were plated as specified above and treated with 0, 0.1, and 1 μM retinol with and without 10-fold molar excess of the RXR antagonist, PA452 (generously provided by Dr. Hiroyuki Kagechika, Tokyo Medical and Dental University, Tokyo, Japan) (162). To measure the effect of a RXR agonist on β-catenin protein levels, all three cell lines were plated as specified above and treated with 0, 1, and 10 μM 9-*cis*-RA. In experiments with proteasome inhibitors, RXR antagonist, and RXR agonists, cells were harvested 24 h after treatment and western immunoblot analysis for β-catenin and β-actin was performed as described.

To determine if retinol treatment alters RXR protein levels, cells were plated as specified above and treated with 0, 1, and 10 μM retinol for 24 h. Protein was harvested as described. Immunoblot analysis utilized RXRα (Santa Cruz Biotechnology, D-20 catalogue #sc-553, Santa Cruz, CA), RXRβ (Santa Cruz Biotechnology, C-20 catalogue #sc-831, Santa Cruz, CA), or RXRγ (Santa Cruz Biotechnology, Y-20 catalogue #sc-555, Santa Cruz, CA) antibodies at a

dilution of 1:1000. After incubation with the corresponding secondary antibody, immunoreactivity was detected as stated above. Membranes were then striped and probed for β-actin. Densitometry was performed using a BioRad Gel Documentation System (Hercules, CA).

Transfections and Luciferase Reporter Assays

To further examine the role of RXRs in β-catenin degradation, cells were plated in 12-well plates at a density of 1 x 10^5 cells/well and incubated overnight in FBS-supplemented medium. The following day HCT-116 and WiDR cells were transfected with 100 pM RXRα silencer validated siRNA (Ambion, Inc., ID: 4476, Austin, TX) or a corresponding negative control siRNA (Ambion, Inc., ID: 4611G, Austin, TX) and 2 μg of pSV- β- gal using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. SW620 cells were transfected with 300 pmol RXRα silencer validated siRNA or a corresponding negative control. Twenty-four hours later the transfection medium was removed and the cells were treated with fresh medium containing 0 and 10 μM retinol for 24 h. RXRα and β-catenin protein levels were determined using western immunoblot analysis as described above. β-Galactosidase activity (β-Galactosidase Enzyme Assay System, Promega, Madison, WI) was used to correct for transfection efficiency.

To examine the effect of retinol on β-catenin/TCF/LEF signaling, cells were transiently transfected with the TCF/LEF luciferase reporter constructs TOPFlash and FOPFlash (Upstate Biotechnology, Waltham, MA). The TOPFlash reporter construct contains multiple optimal TCF/LEF binding sites that, when activated, induce the transcription of a luciferase reporter gene. FOPFlash contains a mutated TCF/LEF binding site that cannot be activated by β-catenin. This vector was used as a negative control. Cells were plated as described above for transfections. The following day cells were transfected using Lipofectamine 2000 according to manufacturer's

protocol with 2 μg of TOPFlash or FOPFlash and 2 μg of pSV-β-gal. Twenty-four hours later the transfection medium was removed and the cells were treated with fresh medium containing 0, 1, and 10 μM retinol for 24 and 48 h. After collection, cells were assayed for β-galactosidase activity (β-Galactosidase Enzyme Assay System, Promega, Madison, WI). Luciferase activity was measured using the Dual Luciferase Kit (Promega, Madison, WI) as per manufacturer's instructions. Luciferase values were normalized to vehicle control treated cells transfected with TOPFlash and corrected for transfection efficiency using β-galactosidase activity.

Quantitative Real-Time RT-PCR Analysis

Quantitative real-time RT-PCR was performed to determine if retinol decreases β-catenin gene expression. HCT-116, WiDr, and SW620 cells were treated with 0, 1, and 10 μM retinol for 24 h. RNA was harvested and isolated using RNAStat (Tel-Test, Friendswood, TX). Two μg of RNA were treated with DNase using the RNase free DNase kit (Promega, Madison, WI) then reverse transcribed utilizing the Reverse Transcription System (Promega, Madison, WI) as per manufacturer's instructions. Quantitative real time-PCR was performed with SYBR Green dye (Perkin-Elmer-Applied Biosystems, Foster City, CA) using an ABI 7900HT (Perkin-Elmer-Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Briefly, an initial denaturation step (10 min at 95°C) was followed by two-step PCR (15 s at 95°C; 1 min at 60°C, 40 cycles). Relative amounts of β-catenin cDNA were analyzed using the ΔΔCt method. Primers for β-catenin and the internal control gene GAPDH were as follows: β-catenin forward primer 5'-CCG CAT GGA AGA AAT AGT TGA AG-3', β-catenin reverse primer 5'-CAA TTC GGT TGT GAA CAT CCC-3', generating a 71-bp product (Serinsoz); GAPDH forward primer: 5'-GCT CAG ACA CCA TGG GGA AGG TG-3' reverse, 5'-CAG CGC CAG CAT CGC CCC ACT TG resulting in a 87-bp product (163).

Quantitative real-time RT-PCR was also used to examine the effect of retinol on cyclin D1 and c-myc gene expression. HCT-116, WiDr, and SW620 cells were serum-starved for 24 h and treated with 1 μg per ml aphidocholine to induce cell cycle synchronization for another 24 h before being incubated with 0, 1, and 10 μM retinol in the presence of 10% FBS for 48 h as described previously (158). RNA was harvested and cDNA was amplified as specified above. Primers for cyclin D1 and c-myc are as follows: cyclin D1 forward primer 5'-CCG TCC ATG CGG AAG ATC-3', reverse primer 5'-ATG GCC AGC GGG AAG AC-3' resulting in a 86-bp product (164); c-myc forward primers 5-ACC ACC AGC AGC GAC TCT GA-3', reverse primer 5'-TCC AGC AGA AGG TGA TCC AGA CT-3' (165). All RT-PCR products were sequenced to confirm identity. The quantitative RT-PCR results are shown relative to vehicle control and corrected for GAPDH levels.

Data Analysis

Statistical analyses were performed using Excel (XP 2002; Microsoft). Two-tailed, paired student's t-tests were performed to test for differences between treatments. Data are expressed as means ± SEM, n=3. Differences were considered significant at $P < 0.05$.

RESULTS

Retinol Decreases β-Catenin Protein Levels

To determine the effect of retinol on total cellular β-catenin protein levels, three ATRA-resistant colon cancer cell lines were treated with increasing concentrations of retinol. The concentrations of retinol used to treat the cells reflected serum retinol concentrations which range from 0.5 to 2 μM (131). A sub-physiological concentration of retinol was represented by 0.1 μM and 1 μM represented a physiological concentration of retinol. The highest level, 10 μM retinol, was used as a pharmacological, but potentially therapeutically relevant, concentration.

Each cell line examined contained a mutation in a β-catenin degradation pathway. The HCT-116 cell line expressed wt p53 and APC, but was heterozygous for phosphorylation-resistant β-catenin (deletion of codon 45: CTNNB1$^{WT/\Delta45}$) (118). The WiDr cell line contained wt APC and β-catenin but mutant p53 (R273H) (121) and the SW620 cell line expressed wt β-catenin, mutant p53 (R273H), and were APC null (119). All three ATRA-resistant colon cancer cell lines exhibited a significant reduction in total cellular β-catenin protein levels due to treatment with 10 μM retinol regardless of mutation after 24 and 48 h (Figure 3.1). The multiple bands displayed in the β-catenin western blots may be due to degradation, ubiquitination, or non-specific binding and have been reported previously (96,105,160,166-168).

In contrast to retinol, treatment with ATRA did not decrease β-catenin protein levels to as great an extent, if at all. In fact, ATRA increased β-catenin protein levels in SW620 cells. ATRA has also been shown to increase β-catenin protein levels in SH-SY5Y neuronal cells and cultured human prostate epithelial cells (169,170). The three cell lines used in the present study are ATRA-resistant and therefore do not exhibit a decrease in cell growth due to ATRA treatment (158). The growth of these cells is decreased by retinol independent of ATRA and RAR. In addition, SW620 cells do not metabolize retinol to ATRA and HCT-116 and WiDr cells produce only small amounts of ATRA, which does not alter RARE-mediated gene expression (158). Because the objective of the present study was to examine the effect of retinol, the form of vitamin A present in the intestinal lumen, on β-catenin protein levels, the mechanism through which ATRA either does not effect or increases β-catenin protein levels was not explored further.

Retinol Increases β-Catenin Ubiquitination and Proteasomal Degradation

Cytoplasmic β-catenin levels are regulated by ubiquitination and subsequent proteasomal degradation. Retinol significantly increased β-catenin ubiquitination in each cell line (Figure 3.2). Anti-ubiquitin antibody was detected at ~ 100 kDa, the size of mono-ubiquitinated β-catenin. Higher molecular weight bands of poly-ubiquitinated β-catenin were also detected at ~170 kDa and ~200 kDa in the HCT-116 and SW620 cell lines and ~120 kDa and ~200 kDa in the WiDr cell line.

To determine if the decrease in total cellular β-catenin in response to retinol treatment was due to increased proteasomal degradation, all three ATRA-resistant colon cancer cell lines were treated with the proteasomal inhibitor, MG132. MG132 is a reversible, selective aldehyde inhibitor of cysteine and serine proteases that enters cells rapidly and reversibly (171). Treatment with MG132 blocked the retinol-induced decrease in β-catenin protein in all three cell lines indicating that retinol decreases β-catenin via proteasomal degradation (Figure 3.3). For example, after 24 h, HCT-116, WiDr, and SW620 cells treated with 10 μM retinol and 1 μM MG132 showed a significant increase in β-catenin protein levels when compared to cells treated with 10 μM retinol alone (Figure 3.3).

To confirm the ability of MG132 to block the effect of retinol on β-catenin protein levels, all three cell lines were also treated with retinol and 0.1 μM MG262, a more selective and irreversible inhibitor of proteasome degradation. Co-treatment with retinol and MG262 also blocked the ability of retinol to decrease total β-catenin protein levels (data not shown). In addition, co-treatment of all three cell lines with retinol and 0.1 μM of the lysosomal inhibitor, Bafilomycin A1, or retinol and 10 μg/ml cycloheximide, failed to block the ability of retinol to decrease total intracellular β-catenin levels (data not shown). These data indicate that retinol reduced β-catenin protein levels by increasing the ubiquitination and subsequent proteasomal

degradation of β-catenin. Retinol did not affect β-catenin protein levels via a lysosomal pathway or a mechanism involving protein translation.

Quantitative real-time RT-PCR was used to determine if retinol treatment alters β-catenin mRNA levels. Treatment of HCT-116 cells with 10 μM retinol for 24 h increased β-catenin mRNA levels slightly when compared to control but this increase was not significant (Figure 3.4A). Retinol treatment did not affect β-catenin mRNA levels at any concentration in WiDr and SW620 cells (Figures 3.4 B and C). Therefore, retinol treatment decreased β-catenin protein levels by increasing the proteasomal degradation of this protein, rather than by decreasing β-catenin gene transcription.

The RXR-Mediated Degradation Pathway May Facilitate the Retinol-Induced Degradation of β-Catenin

Multiple pathways direct β-catenin to the proteasome for degradation including a Wnt/GSK-3β/APC, p53/Siah-1/APC, and a RXR-mediated pathway. The RXR pathway is the only fully functional pathway in all three cell lines. The RXR isoforms RXRα, -β, and -γ are present in all three ATRA-resistant colon cancer cell lines after 24 h of retinol treatment (Figure 3.5). The HCT-116 cell line exhibited a significant increase in RXRα and RXRγ protein levels with retinol treatment. WiDr cells displayed a significant increase in RXRα in response to retinol treatment. SW620 cells tended to increase in RXRα ($P = 0.09$) protein levels and exhibited a significant increase in RXRγ protein in response to retinol treatment. In contrast, retinol treatment did not significantly change RXRβ protein levels in any of the three cell lines.

To determine if retinol decreases β-catenin levels through the RXR-mediated degradation pathway, cells were treated with retinol and a 10-fold molar excess of PA452, a RXR-selective antagonist (162). Treatment with 10 μM retinol was not examined in this study because 100 μM

PA452 proved toxic to the cells. After 24 h, HCT-116, WiDr, and SW620 cells showed a decrease in β-catenin with retinol treatment. The RXR antagonist blocked the ability of retinol to decrease β-catenin protein levels in the HCT-116 cell line (Figure 3.6A). A similar trend (P = 0.06) was exhibited by the WiDr and SW620 cell lines (Figure 3.6 B and C).

To provide additional support that RXRs facilitate the ability of retinol to induce the proteasomal degradation of β-catenin, all three cell lines were transfected with RXRα silencing siRNA. Transfection with RXRα siRNA significantly decreased RXRα protein levels in all three cell lines (Figure 3.7). This reduction in RXRα protein hindered the ability of retinol to decrease β-catenin protein levels (Figure 3.7), particularly in the SW620 cell line that expressed mutant p53 and was APC null (Figure 3.7C).

To determine if a RXR agonist alters β-catenin protein levels, cells were treated with 9-*cis*-RA. After 24 h, 9-*cis*-RA had no effect on β-catenin protein levels in any cell line (Figure 3.8) (162). Treatment with PA024, a synthetic RXR agonist, also did not affect β-catenin protein levels (data not shown). The ability of a RXR antagonist and RXRα siRNA to block the retinol-induced decrease in β-catenin in all three cell lines indicates that the RXR-mediated pathway may facilitate the proteasomal degradation of β-catenin due to retinol treatment. The failure of RXR agonists to mimic retinol's ability to decrease β-catenin protein suggests the RXR-mediated pathway promotes β-catenin degradation independent of RXR-ligand binding.

Retinol Treatment Decreases β-Catenin-Mediated Gene Transcription

To determine if retinol treatment could reduce β-catenin-mediated gene transcription, cells were transiently transfected with the TCF/LEF luciferase reporter construct, TOPFlash (Upstate Biotechnology, Waltham, MA). Luciferase activity was significantly decreased after 48 h of retinol treatment in all three ATRA-resistant colon cancer cell lines (Figure 3.9). HCT-116

cells treated with 10 μM retinol showed a 20% reduction in luciferase activity, while the WiDr and SW620 cells displayed an approximately 50% decrease in luciferase activity. Therefore, the retinol-induced decrease in total cellular β-catenin resulted in reduced β-catenin-mediated transcription of an exogenous gene containing TCF/LEF binding sites.

The β-catenin/TCF/LEF complex induces the transcription of genes involved in cell proliferation (e.g. cyclin D1 and c-myc) (77,78). Because the cyclin D1 and c-myc promoters contain TCF/LEF binding sites (77), quantitative real-time RT-PCR was used to determine if retinol treatment affected cyclin D1 and c-myc mRNA levels. Retinol significantly decreased cyclin D1 mRNA levels in the WiDr and SW620 cell lines but not in the HCT-116 cell line (Figure 3.10 A-C). These data support our previous study showing that retinol inhibits cell growth by inducing $G_{0/1}$ cell cycle arrest in WiDr and SW620 cells but not HCT-116 cells (158). In addition, retinol treatment significantly decreased c-myc mRNA levels in all three ATRA-resistant colon cancer cell lines (Figure 3.10 D-F). These data indicate that the retinol-induced decrease in total β-catenin correlates with decreased transcription of endogenous targets of β-catenin that mediate tumor growth.

DISCUSSION

This study shows that retinol decreases β-catenin protein levels in ATRA-resistant human colon cancer cell lines despite mutations in the p53 and APC proteins that regulate β-catenin protein degradation. Retinol decreases β-catenin protein levels by increasing the ubiquitination and proteasomal degradation of this protein, rather than by decreasing β-catenin gene transcription. The ability of a RXR antagonist and RXRα siRNA to hinder the retinol-induced decrease in β-catenin indicates that the recently discovered RXR degradation pathway (96) may facilitate the proteasomal degradation of β-catenin by retinol. RXR agonists did not mimic the

ability of retinol to decrease β-catenin protein levels suggesting the RXR-mediated degradation pathway is active in the absence of a ligand. Treatment of ATRA-resistant colon cancer cells with retinol also results in decreased transcription of the exogenous TCF/LEF reporter construct, TOPFlash, as well as the endogenous β-catenin target genes, cyclin D1, in the WiDr and SW620 cell lines, and c-myc, in all three cell lines. We have previously shown that retinol decreases ATRA-resistant colon cancer cell growth by slowing the progression from the $G_{0/1}$ to the S phase of the cell cycle (158); therefore, we hypothesize that retinol inhibits the growth of ATRA-resistant colon cancer cell lines by decreasing total intracellular β-catenin levels via a mechanism involving ligand-independent RXR-mediated proteasomal degradation, ultimately resulting in decreased transcription of genes involved in tumor progression.

To our knowledge, the effects of retinol on β-catenin have not been previously examined *in vitro*, however some *in vivo* studies point to a link between dietary vitamin A, colon cancer and β-catenin. For example, a recent study by Delage et al (34) showed that dietary supplementation with retinyl palmitate reduced the occurrence of carcinogen and high fat diet-induced aberrant crypt foci. Because retinyl palmitate is converted to retinol in the intestinal lumen, the colonocytes of these rats were exposed to retinol. Supplementation with retinyl palmitate also prevented the increase in colonocyte β-catenin due to consumption of a high fat diet (159), indicating that retinol may decrease β-catenin protein levels *in vivo* as well as *in vitro*. In human patients, cellular retinol binding protein (CRBP)-I levels were decreased in hepatocellular carcinomas (172). Reduced CRBP-I levels were also associated with a lower two-year survival rate (172). Intracellular retinol storage and transport are mediated by CRBPs. Interestingly, nuclear CRBP-I inclusions were co-localized with nuclear β-catenin, indicating a potential cross-talk between β-catenin and CRBP-I, and potentially retinol (172).

Retinoid receptors either decrease β-catenin protein levels, as in the case of RXR, or inhibit β-catenin-mediated gene transcription, as in the case of RAR. For example, Xiao et al., (96) showed that the proteasomal degradation of β-catenin could be induced in an APC-independent manner by RXR. *In vitro*, RXRα interacted with β-catenin. This interaction did not require the presence of a synthetic RXR agonist (96), indicating that RXRs can mediate β-catenin degradation in the absence of a ligand. It has also been reported that RXR protein is marked for degradation upon ligand binding (173); therefore, the presence of a RXR ligand would lead to a decrease in RXR protein. In contrast, our study shows that retinol, which is not an RXR ligand, increases RXRα and RXRγ protein levels (Figure 3.5) concomitant with a reduction in β-catenin protein levels (Figure 3.1). We also demonstrate that the RXR antagonist, PA452, and RXRα siRNA inhibit the ability of retinol to decrease total cellular β-catenin levels in all three cell lines (Figure 3.6 and 3.7, respectively). RXRα siRNA was chosen because all three cell lines show an increase in RXRα protein in response to retinol treatment and previous studies have focused primarily on RXRα (96,174-177). RXRβ protein was not significantly altered with retinol treatment; therefore, silencing RXRβ may provide no further insight into the RXR-mediated degradation of β-catenin. RXRγ protein also increased in the HCT-116 and SW620 cell lines in response to retinol treatment, but, to our knowledge, a validated RXRγ siRNA is not available at this time.

Although retinol does not directly bind RXR, additional evidence that retinol may reduce β-catenin protein levels via a RXR-mediated degradation pathway is exhibited by all three ATRA-resistant colon cancer cell lines, each of which contains at least one nonfunctional β-catenin degradation pathway. For example, HCT-116 cells are heterozygous for phosphorylation-resistant β-catenin. Without the ability to efficiently phosphorylate β-catenin

the Wnt/GSK-3β/APC pathway alone could not effectively reduce β-catenin to the very low levels reached with the 10 μM retinol treatment because half of the β-catenin present in the cells cannot be phosphorylated (Figure 3.1). Therefore, HCT-116 cells most likely utilize the p53/Siah-1/APC, the RXR-mediated degradation pathway, or a combination of the two to achieve the significant decrease seen in β-catenin due to retinol treatment. In addition, the WiDr cells contain mutant p53, yet decrease β-catenin protein in response to retinol treatment (Figure 3.1). Without the capability to induce the p53/Siah-1/APC pathway, the WiDr cells degrade β-catenin using the Wnt/GSK-3β/APC or the RXR-mediated degradation pathway. Finally, the SW620 cell line contains mutant p53 and is APC null. Without functional p53 the SW620 cells cannot effectively use the p53/Siah-1/APC pathway. In addition, lack of APC renders the Wnt/GSK-3β/APC pathway inefficient, yet retinol markedly decreased β-catenin protein levels in this cell line.

The Hakai pathway can also facilitate the proteasomal degradation of β-catenin. β-Catenin binds directly to E-cadherin at the cell membrane. Hakai, an E3-ubiquitin-ligase, binds to E-cadherin promoting the ubiquitination and subsequent proteasomal degradation of E-cadherin and the β-catenin bound to E-cadherin [reviewed in (178)]. Thus, if the Hakai pathway was active E-cadherin ubiquitination would increase and E-cadherin levels may also decrease. However, neither E-cadherin ubiquitination nor E-cadherin protein levels were affected by retinol treatment (data not shown). Therefore, we believe that the RXR-mediated degradation pathway, the one pathway functional in all three cell lines, may facilitate the proteasomal degradation of β-catenin.

In contrast to the cell lines used in the current study, Byers et al (116) demonstrated that 9-*cis*-RA treatment increased β-catenin protein levels in SKBR3 breast cancer cells by increasing

β-catenin protein stability (116), perhaps due to the ability of RXR ligands to decrease RXRα-mediated nuclear export. 9-*Cis*-RA treatment did not increase β-catenin levels in CaCo2 cells (105). In CaCo2 cells, 9-*cis*-RA treatment induced epithelial differentiation characterized by an increase in cadherin expression in regions of cell to cell contact (117). The effects of 9-*cis*-RA on SKBR3 cells were mediated by RARα, not RXR. 9-*Cis*-RA also inhibited β-catenin mediated gene transcription via TCF/LEF in the ATRA-sensitive cell lines MCF-7, HS578t, CaCo2, and HT-29 cells (105). However, in each of these cell lines, the effect of 9-*cis*-RA was mediated by RAR. 9-*Cis*-RA-bound RAR decreased β-catenin-mediated gene transcription by competing with TCF for β-catenin binding (105). In addition, the effects of 9-*cis*-RA on β-catenin-mediated gene transcription differ depending on the level of endogenous β-catenin (160). For example, in SKBR3 cells, which express very low levels of β-catenin, transcription via a cyclin D1 promoter was regulated by AP-1. However, in SW480 cells, which express high endogenous levels of β-catenin, similar to the cell lines used in the present study, the effect of 9-*cis*-RA on the transcription of the same cyclin D1 promoter was regulated by TCF, not AP-1. These data indicate that β-catenin levels may control cyclin D1 transcription in colon cancer cell lines expressing high levels of β-catenin, such as those used in the current study.

The SW620 and WiDr cell lines exhibit a decrease in cyclin D1 levels due to retinol treatment associated with a decrease in total cellular β-catenin protein levels (Figures 3.10 and 3.1, respectively). Previously, we showed that retinol, but not ATRA, decreases SW620 and WiDr cell growth by inducing $G_{0/1}$ cell cycle arrest not cellular differentiation, apoptosis, or necrosis (158). Retinol was not metabolized to bioactive compounds in SW620 cells, while a small amount of ATRA was made in the HCT-116 and WiDr cells (158). This small amount of ATRA was unable to activate transcription via a RARE-CAT reporter construct (158). Also, 9-

cis-RA was not synthesized from retinol in any cell line. Importantly, a RAR antagonist was unable to block the ability of retinol to decrease the growth of the cell lines, indicating that retinol, unlike 9-*cis*-RA, acts independent of the RAR to affect cell growth. Because the three colon cancer cell lines used in the current study are ATRA-resistant, lacking some or all RAR (42,56,120,160,161), and do not synthesize 9-*cis*-RA or large amounts of ATRA from retinol, retinol does not affect β-catenin protein levels or β-catenin mediated gene transcription via a mechanism involving RAR.

To our knowledge, no mechanistic studies have been performed to show how RXRs regulate β-catenin degradation. However, RXRα and β-catenin have been shown to directly interact (110). RXRα also contains a putative nuclear export signal in its carboxyl-terminal region (179). Therefore, we hypothesize that the increase in RXRα levels in response to retinol treatment increases the movement of RXRα/β-catenin complexes out of the nucleus, to the cytoplasm for proteasomal degradation. Interestingly, RXR ligands suppress RXRα's nuclear export activity (179), supporting our finding that the degradation of β-catenin is not induced by the RXR ligand, 9-*cis*-RA. This RXR-mediated pathway may also involve multi-protein complexes and multi-step reactions, potentially involving other nuclear receptors, such as PPAR which also binds to the RXRα/β-catenin complex (110). The steps linking retinol to increased RXR protein levels, RXR to β-catenin degradation, and the effect of retinol on the subcellular localization of β-catenin will be explored in future studies.

In conclusion, this study shows that retinol increases the proteasomal degradation of β-catenin in ATRA-resistant human colon cancer cells, decreasing the transcription of both exogenous and endogenous β-catenin target genes. In addition, the degradation of β-catenin may be regulated by the RXRs. We hypothesize that retinol inhibits ATRA-resistant human colon

cancer cell growth by decreasing free nuclear β-catenin protein levels, decreasing the transcription of cyclin D1 and c-myc, ultimately resulting in slowed cell cycle progression. Resistance to ATRA due to loss of RAR expression is a common occurrence during carcinogenesis (30), limiting the effectiveness of ATRA chemotherapy. We speculate that retinol may prove to be a more successful colon cancer chemotherapy than ATRA. Elucidating the pathway by which retinol inhibits the growth of ATRA-resistant colon cancer cells is an important step towards the development of retinoid derived chemotherapies for cancer treatment.

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Figure 1

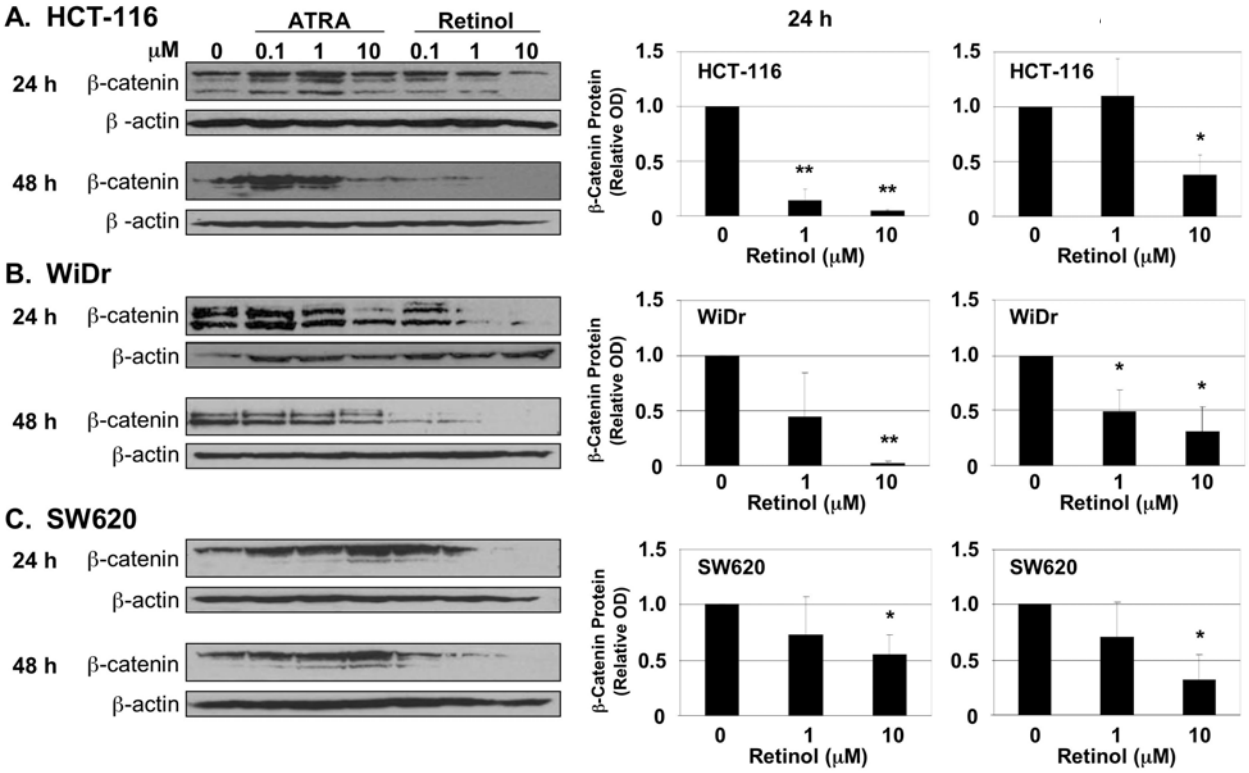

Figure 3.1 Retinol decreases total cellular β-catenin protein levels. (A) HCT-116, (B) WiDr, and (C) SW620 cells were plated, treated with 0, 0.1, 1, and 10 μM ATRA or retinol for 24 or 48 h. Following treatment, the proteins were harvested, electrophoresed and probed with β-catenin and β-actin antibodies as described in Materials and Methods. β-Actin was used to demonstrate equal loading. This experiment was performed three times with similar results; one representative western blot is shown for each time point. Values shown in the graphs are the mean of three separate experiments ± SEM. **P* < 0.05, ***P* < 0.01; significantly different from vehicle control.

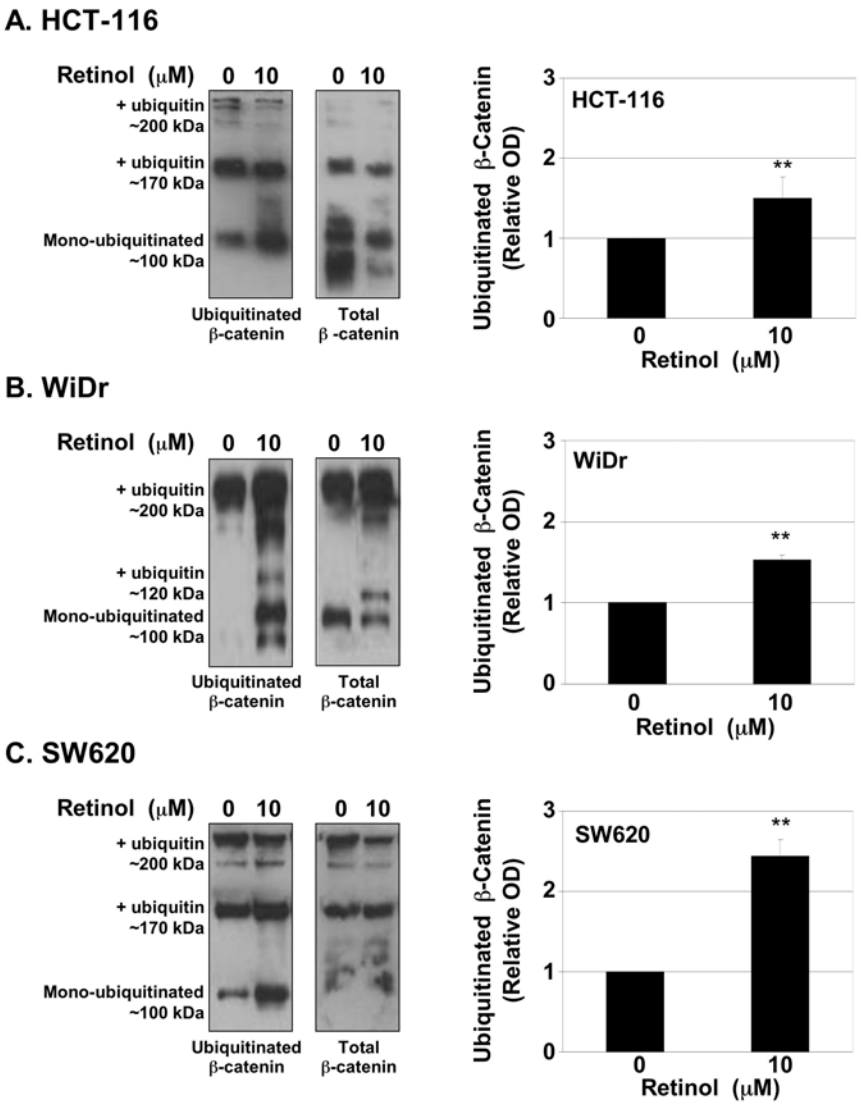

Figure 3.2 Retinol increases β-catenin ubiquitination. (A) HCT-116, (B) WiDr, and (C) SW620 cells were treated with 0 and 10 μM retinol. After 24 h, total protein was harvested, and cell extracts were immunoprecipitated using a β-catenin antibody and immunoblotted for ubiquitin and β-catenin. This experiment was performed three times with similar results; one representative western blot is shown. Ubiquitinated β-catenin was calculated by dividing total ubiquitinated β-catenin by total β-catenin and normalizing to vehicle control. Values shown are the mean of three separate experiments ± SEM. *$P < 0.05$, **$P < 0.01$; significantly different from vehicle control.

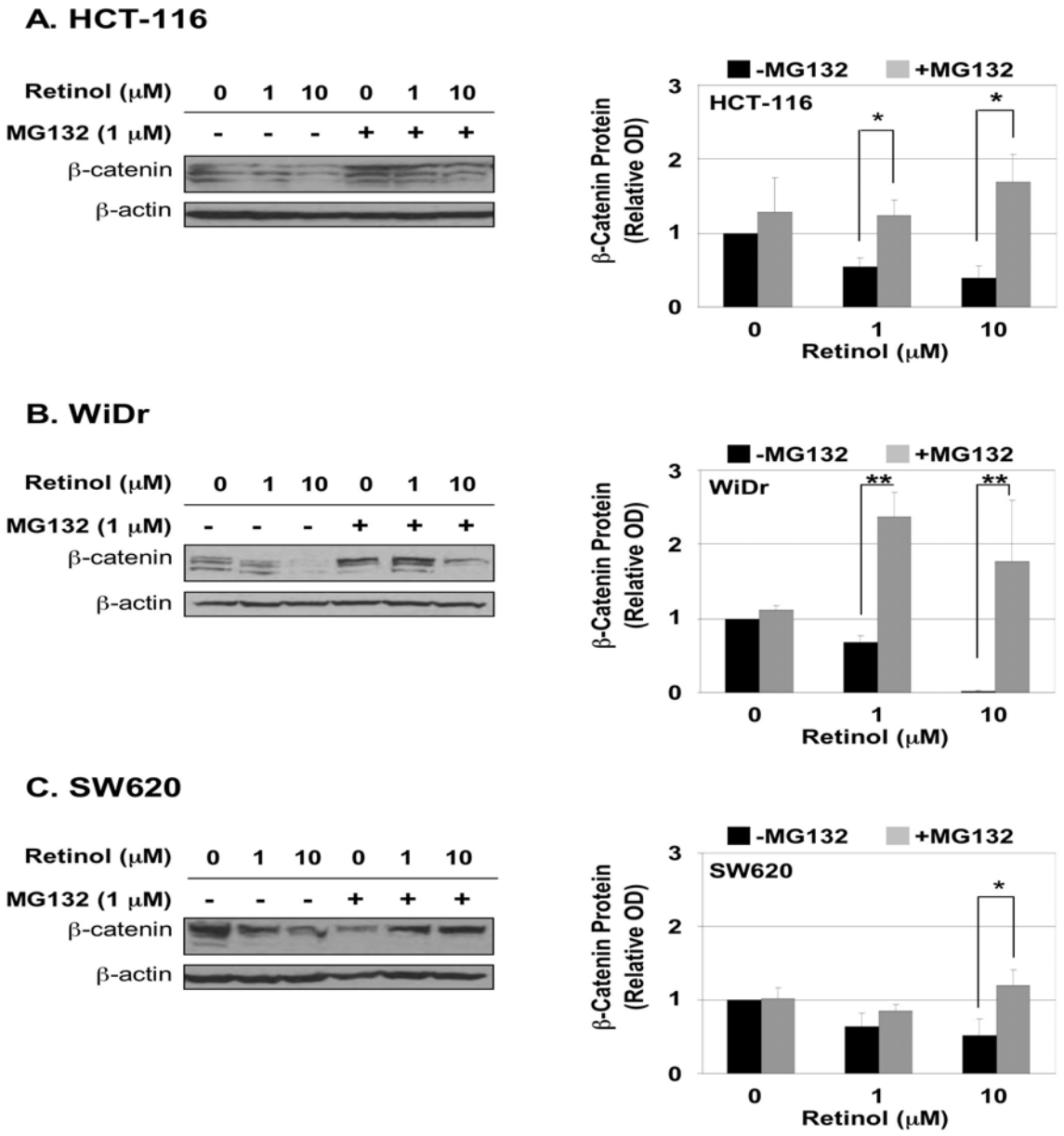

Figure 3.3 Retinol decreases β-catenin through increased proteasomal degradation. (A) HCT-116, (B) WiDr, and (C) SW620 cells were treated with 0, 1, and 10 μM retinol with and without 1 μM of the proteasomal inhibitor MG132. After 24 h, total protein was harvested, electrophoresed and probed for β-catenin and β-actin. This experiment was performed three times with similar results; one representative western blot is shown. Values shown are the mean of three separate experiments ± SEM. **P* < 0.05, ***P* < 0.01; significantly different from cells not treated with MG132.

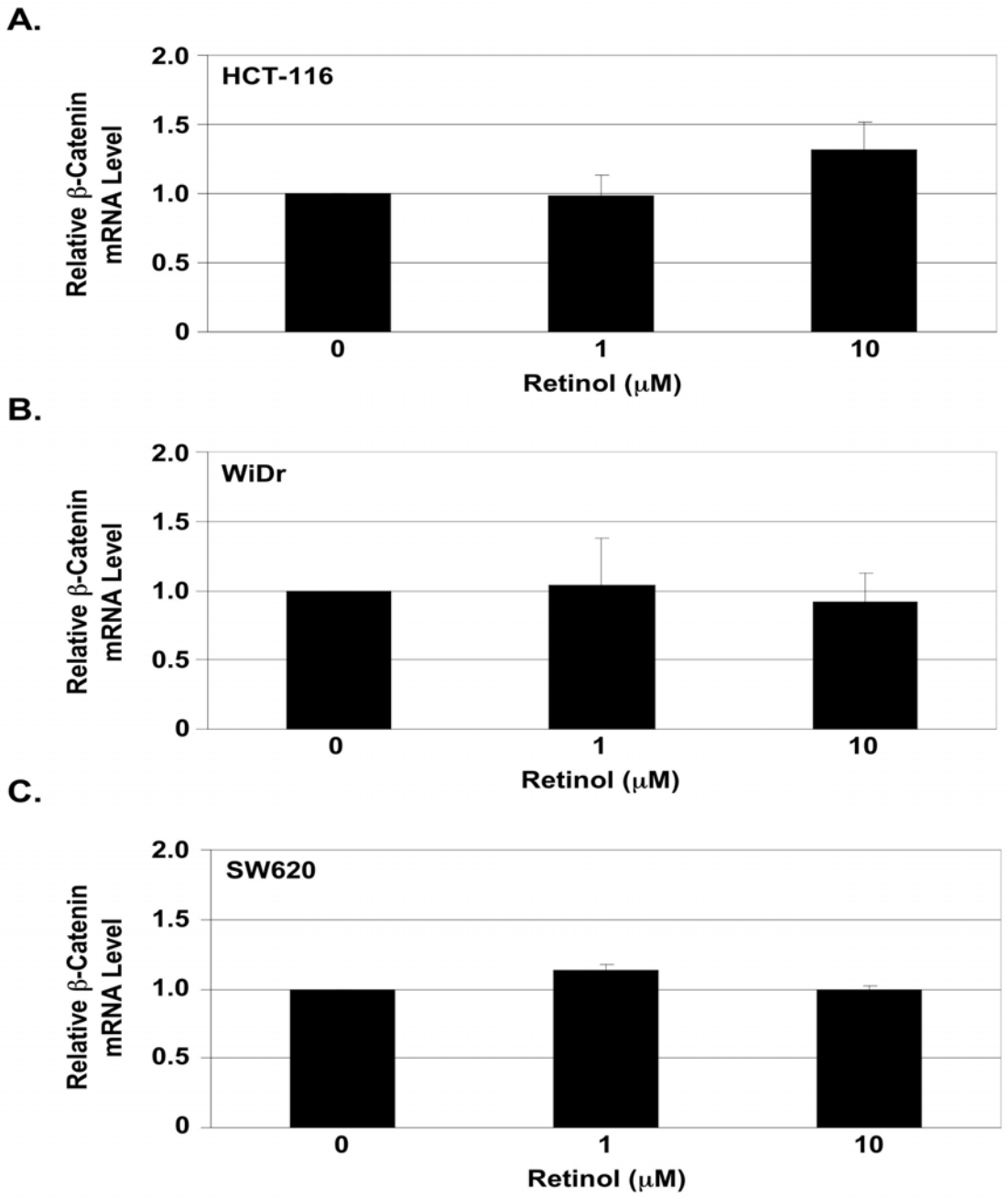

Figure 3.4 Retinol does not decrease β-catenin gene expression. Quantitative real time RT-PCR was performed as described in Materials and Methods. (A) HCT-116, (B) WiDr, and (C) SW620 cells were treated for 24 h with 0, 1, and 10 μM retinol. Total RNA was reverse transcribed using random primers and then amplified on the ABI 7900HT machine (Perkin-Elmer-Applied Biosystems, Foster City, CA) using the SYBR green I Quantitect kit and analyzed using the ΔΔCt method. Experiments were repeated three times and each PCR reaction was run in duplicate. β-Catenin mRNA levels are shown relative to vehicle control and corrected for GAPDH. Values shown are the mean of three separate experiments ± SEM.

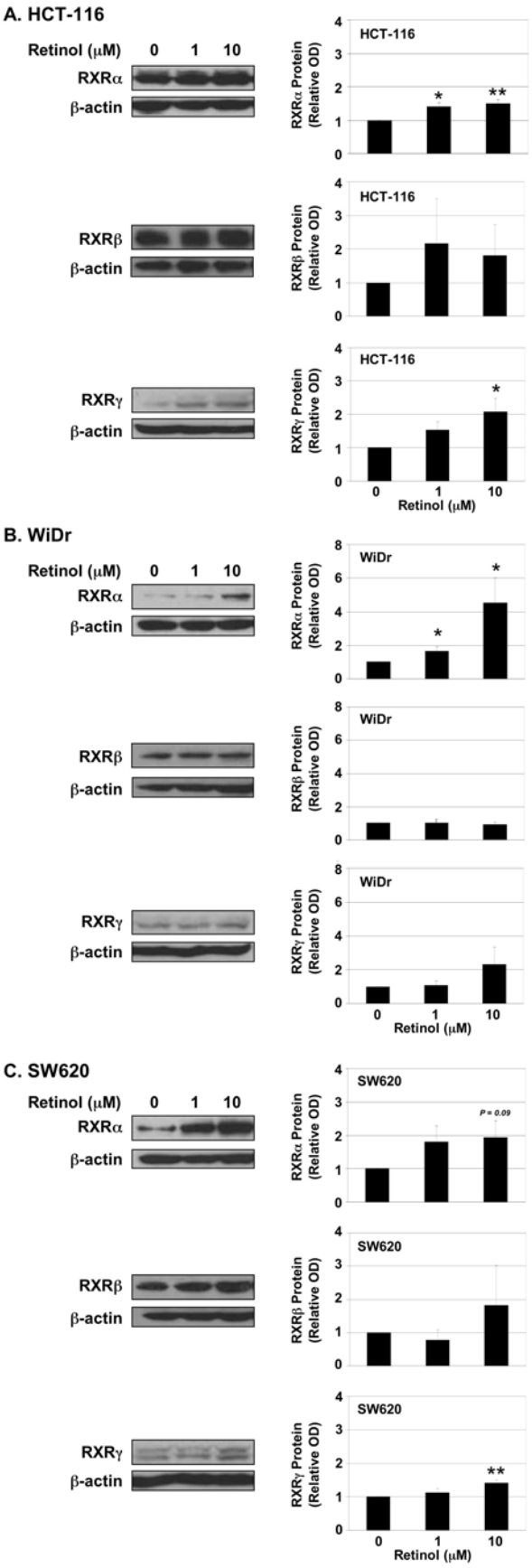
A. HCT-116
Retinol (μM) 0 1 10
RXRα
β-actin
RXRβ
β-actin
RXRγ
β-actin
HCT-116
RXRα Protein (Relative OD)
RXRβ Protein (Relative OD)
RXRγ Protein (Relative OD)
Retinol (μM)
B. WiDr
WiDr
C. SW620
SW620
P = 0.09

Figure 3.5 Retinol increases RXRα and -γ, but not RXRβ, protein levels. RXRα, -β, and -γ protein levels were measured in (A) HCT-116, (B) WiDr, and (C) SW620 cells treated for 24 h with 0, 1, or 10 μM retinol. Total protein was harvested and immunoblotted for RXRα, -β, or -γ and β-actin. This experiment was performed three times with similar results; one representative western blot is shown. Values shown are the mean of three separate experiments ± SEM. **P* < 0.05, ***P* < 0.01; significantly different from vehicle control.

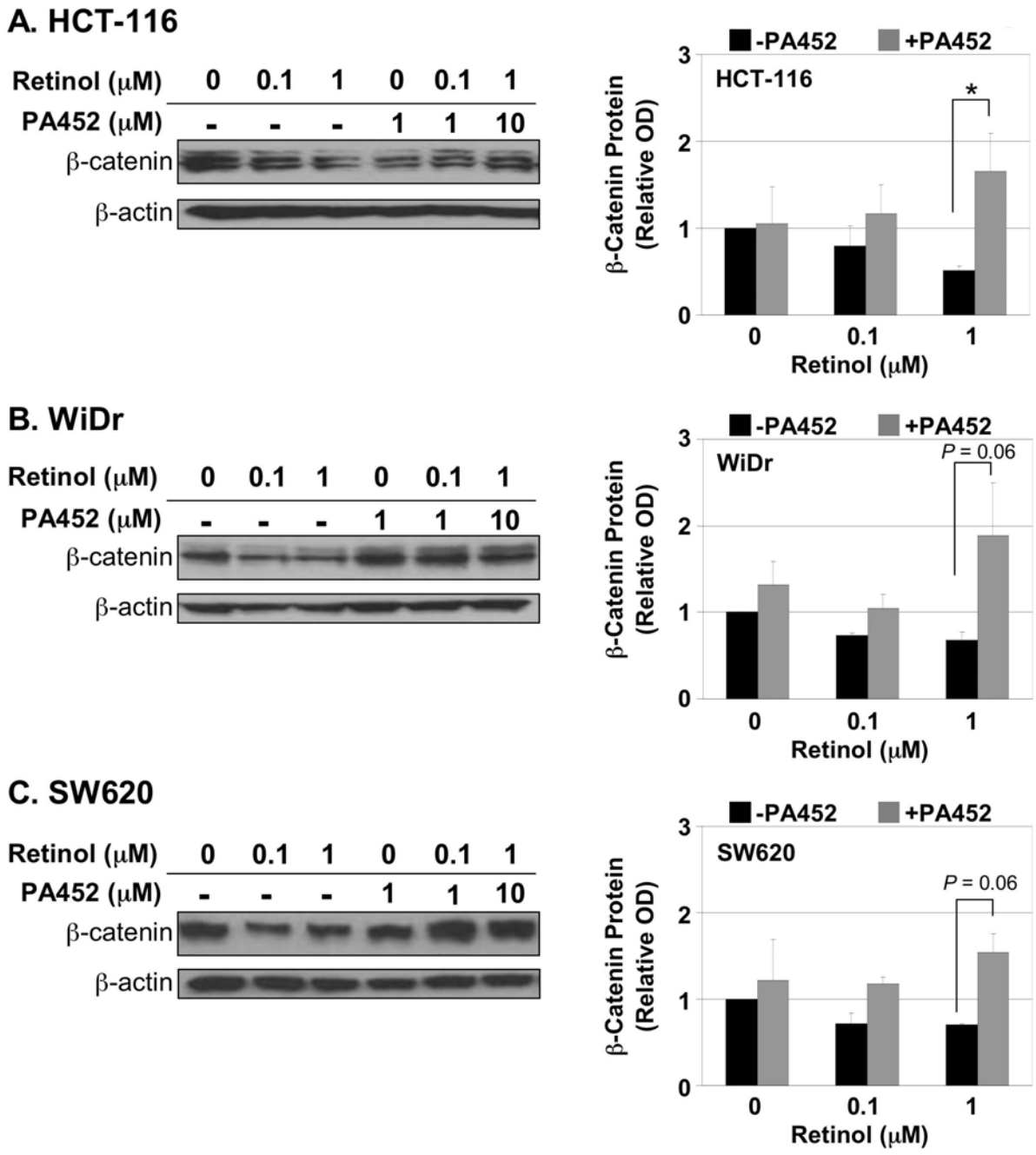

Figure 3.6 A RXR pan-antagonist blocks the retinol-induced decrease in β-catenin. (A) HCT-116, (B) WiDr, and (C) SW620 cells were treated with 0, 0.1, and 1 μM retinol with and without a 10-fold molar excess of PA452. After 24 h, total protein was harvested, electrophoresed and probed for β-catenin and β-actin. This experiment was performed three times with similar results; one representative western blot is shown. Values shown are the mean of three separate experiments ± SEM. *$P < 0.05$, **$P < 0.01$; significantly different from cells not treated with PA452.

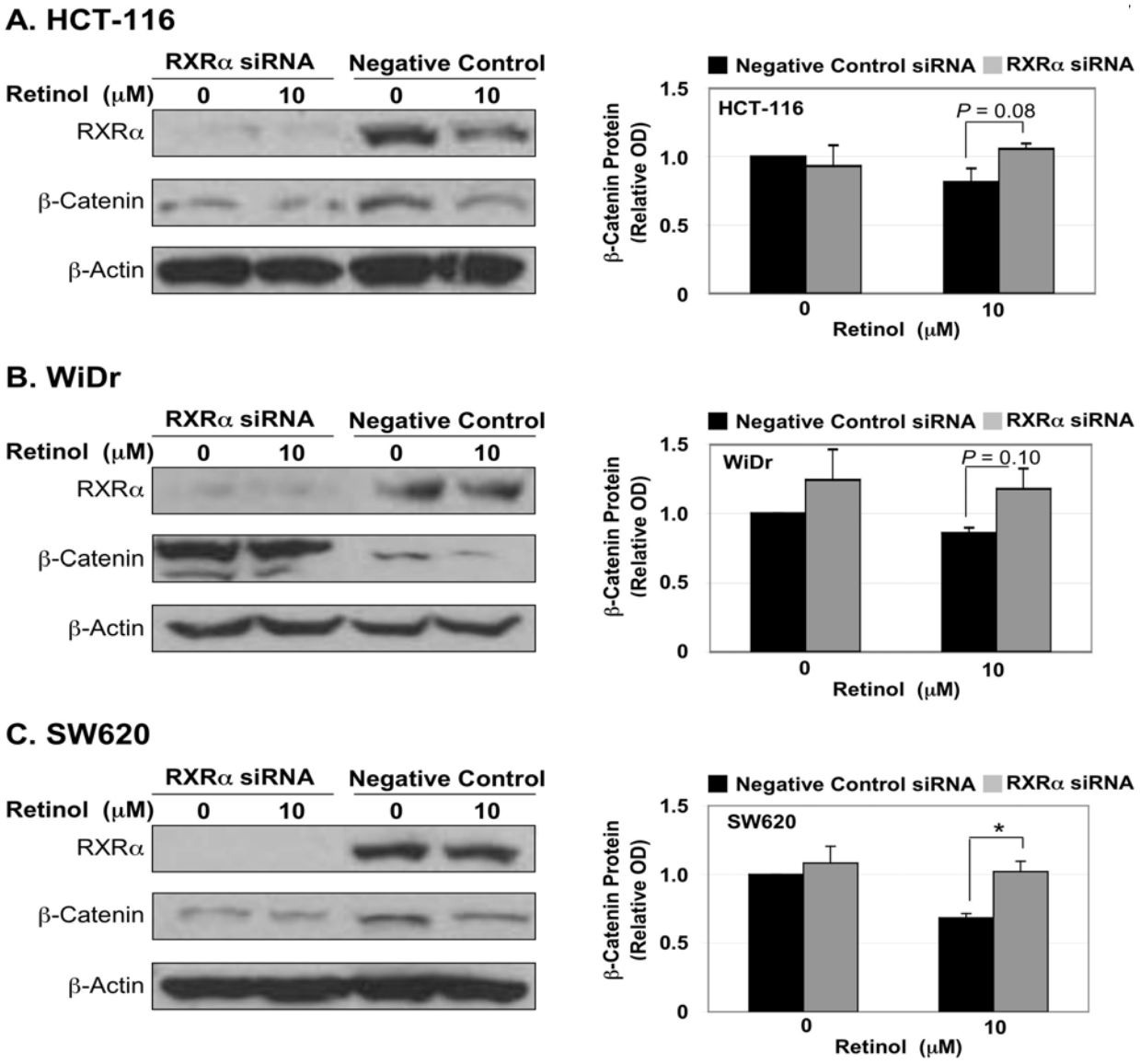

Figure 3.7 RXRα siRNA reduces the ability of retinol to decrease β-catenin protein. RXRα and β-catenin protein levels were determined in (A) HCT-116, (B) WiDr, and (C) SW620 cells transfected with 100 (HCT-116 and WiDR) or 300 (SW620) pmol RXRα siRNA and 2 μg pSV-β- galactosidase using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Twenty-four hours following transfection, cells were treated with 0 and 10 μM retinol. Cells were harvested 24 h after retinol treatment. Total protein was harvested, electrophoresed and probed for RXRα, β-catenin and β-actin. β-Catenin protein levels were normalized for transfection efficiency using β-galactosidase activity and differences in loading were normalized using β-actin. The normalized β-catenin level in cells treated with 0 μM retinol and transfected with negative control siRNA was set equal to one and all other values are expressed as relative OD. This experiment was performed three times with similar results; one representative western blot is shown. Values shown are the mean of three separate experiments ± SEM. *$P < 0.05$; significantly different from cells transfected with negative control siRNA.

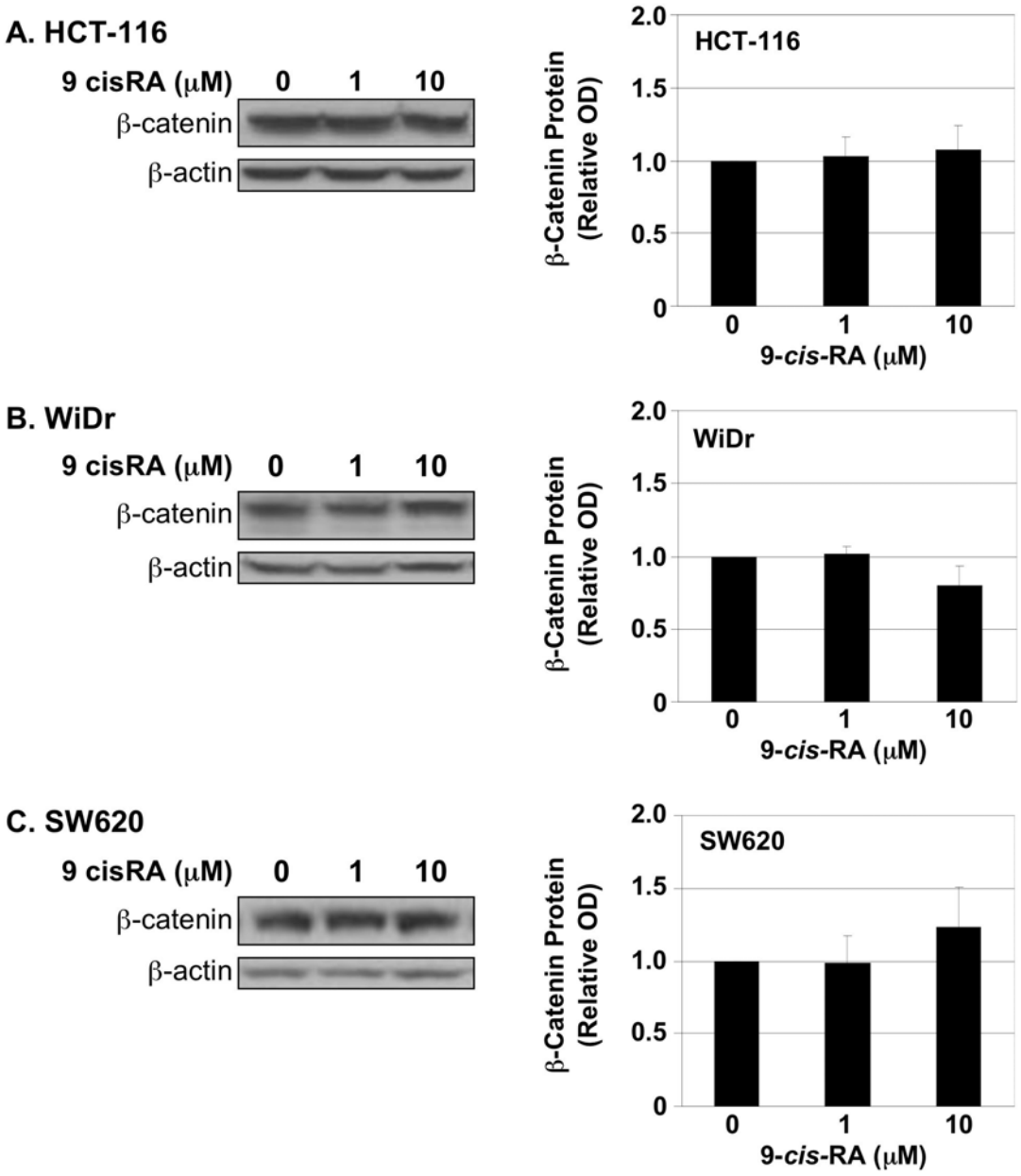

Figure 3.8 RXR agonist treatment does not decrease β-catenin protein levels. (A) HCT-116, (B) WiDr, and (C) SW620 cells were treated with 0, 1, and 10 μM 9-cis-RA. After 24 h of treatment with 9-*cis*-RA, total protein was harvested, electrophoresed and probed for β-catenin and β-actin. This experiment was performed three times with similar results; one representative western blot is shown. Values shown are the mean of three separate experiments ± SEM.

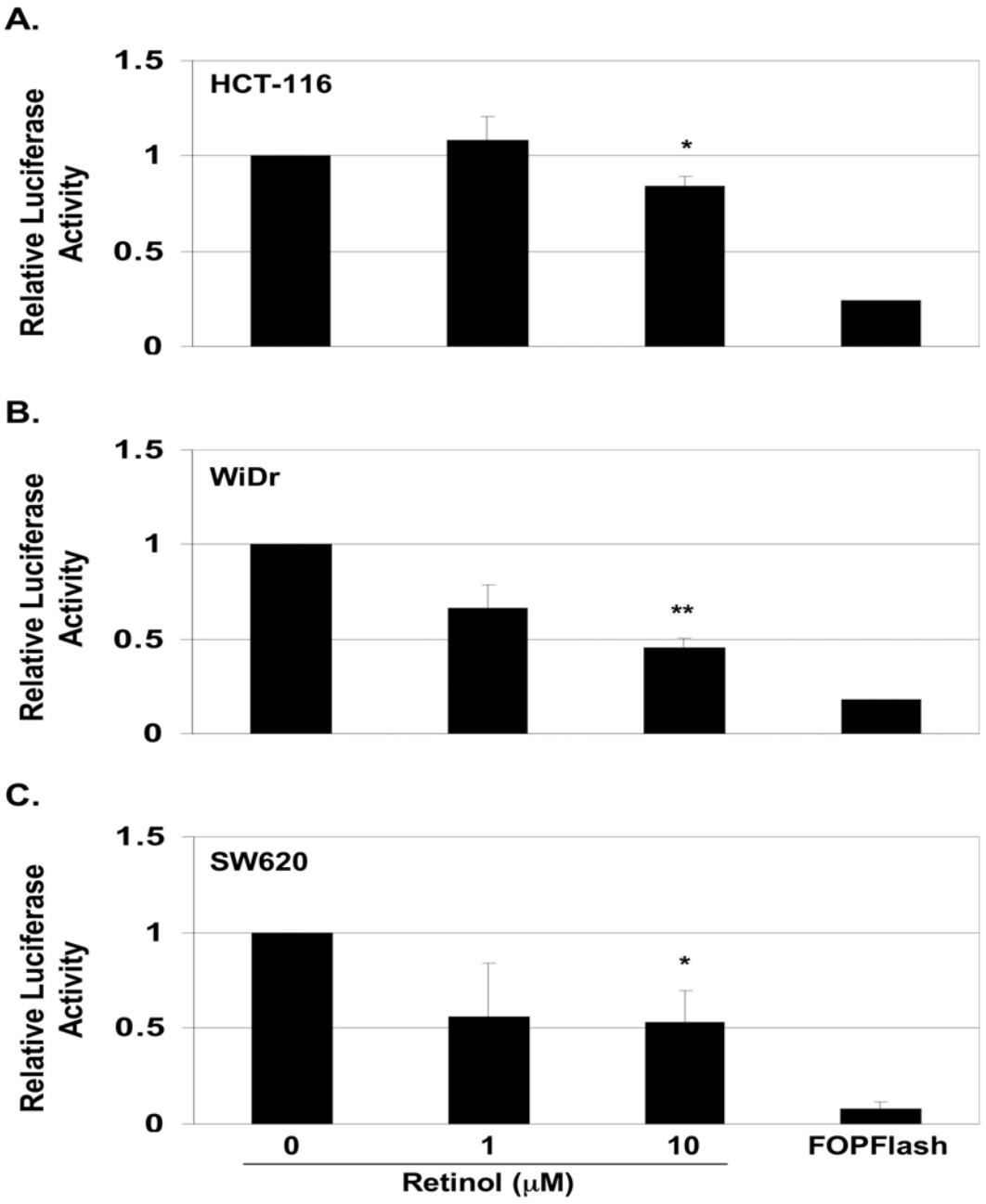

Figure 3.9 Retinol decreases β-catenin-mediated gene transcription. (A) HCT-116, (B) WiDr, and (C) SW620 cells were transiently transfected with 2 μg TOPFlash or 2 μg FOPFlash and 2 μg pSV-β-gal using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Twenty-four hours following transfection, cells were treated with 0, 1, and 10 μM retinol. Cells were harvested 48 h after retinol treatment and luciferase and β-galactosidase activity assays were performed. Luciferase activity was normalized for transfection efficiency using β-galactosidase activity. The normalized luciferase activity in control cells treated with ethanol vehicle was set equal to one and all other values are expressed as relative OD. Values shown are the mean of three separate experiments ± SEM. *$P < 0.05$, **$P < 0.01$; significantly different from vehicle control.

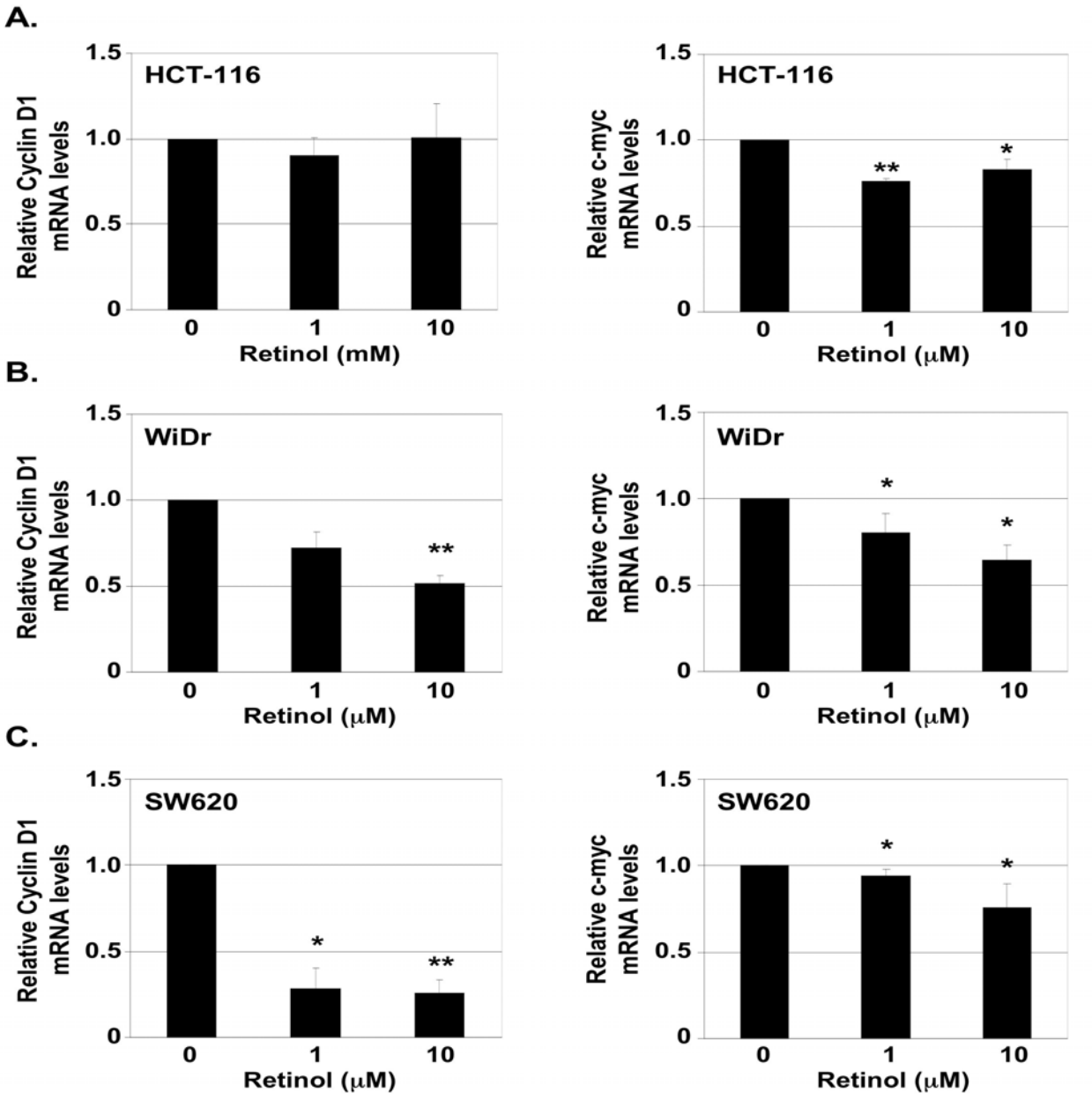

Figure 3.10 Retinol decreases cyclin D1 and c-myc mRNA levels. Quantitative real time RT-PCR was performed as described in Materials and Methods. (A) HCT-116, (B) WiDr, and (C) SW620 cells were synchronized with aphidocholine for 24 h and then treated for 48 h with 0, 1, and 10 μM retinol as described previously (158). Total RNA was reverse transcribed using random primers and then amplified on the ABI 7900HT machine (Perkin-Elmer-Applied Biosystems, Foster City, CA) using the SYBR green I Quantitect kit and analyzed using the ΔΔCt method. Experiments were repeated three times and each PCR reaction was run in duplicate. The results are calculated relative to vehicle control and corrected for GAPDH. Values shown are the mean of three separate experiments ± SEM. **P* < 0.05, ***P* < 0.01; significantly different from control.

Chapter 4: Retinol increases β-catenin-RXRα binding leading to the increased proteasomal degradation of β-catenin and RXRα

ABSTRACT

We previously reported that retinol utilized a RXR-mediated proteosomal degradation pathway to decrease β-catenin protein levels in *all-trans* retinoic acid (ATRA)-resistant human colon cancer cell lines. The current study examines the effects of retinol on the molecular interactions between RXRα and β-catenin in ATRA-resistant human colon cancer cell lines. Our model utilized the HCT-116 and SW620 cell lines treated with 0 or 10 μM retinol for 8, 16, 24 and 48 h. Retinol treatment triggered a relocation of β-catenin and RXRα proteins. Specifically, cells treated with retinol for 8 and 24 h displayed increased cytosolic but decreased nuclear β-catenin and RXRα. Retinol treatment significantly increased β-catenin and RXRα protein interaction by 8 h. Previously we showed that 24 h of retinol treatment increased RXRα protein. Here we show that this increase in RXRα levels is due to increased RXRα mRNA. Also, 48 h of retinol treatment significantly decreased RXRα protein levels. Treatment with a proteasomal inhibitor blocked the decrease in RXRα. Lastly, by transfecting HCT-116 cells with a RXRα construct lacking the AF-1 and DNA binding domains, we show that RXRα and β-catenin binding was required for the proteosomal degradation of β-catenin. Taken together these results suggest that retinol induces RXRα and β-catenin binding and induces their transport to the cytosol where both proteins are proteasomally degraded.

INTRODUCTION

The retinoids, a group of compounds consisting of vitamin A (retinol), its natural metabolites and several synthetic compounds, exhibit inhibitory effects on tumor cell growth [for review please see: (14,30,125)] and regulate gene transcription through two nuclear receptors,

the retinoic acid receptors (RAR) and retinoid X receptors (RXR) (180). Within the cell, retinol can be esterified and stored as retinyl esters or converted to *all-trans* retinoic acid (ATRA). ATRA is the primary ligand for RAR. When, ligand bound, RAR heterodimerize with RXR to regulate the expression of target genes containing retinoic acid responsive elements (RAREs) in their promoter regions. However, cells frequently become resistant to the growth inhibitory effects of ATRA as cancer progresses, usually due to loss of the ability to induce the transcription of RAR in response to ATRA [for review please see: (30)]. Previous work in our laboratory has shown that retinol, the form of vitamin A derived from the diet, can inhibit the growth of ATRA-resistant human colon cancer cell lines by affecting cell cycle progression via a novel ATRA, RAR, and RARE-independent mechanism (158).

Animal-derived food sources contain preformed vitamin A predominantly as retinyl esters. Retinyl esters are cleaved within the intestinal lumen to yield retinol. The diet contains very little ATRA, the most widely studied and bioactive metabolite of retinol (14). Therefore, human colonocytes are exposed primarily to retinol via the intestinal lumen and elevated intraluminal retinol concentrations can be achieved via dietary vitamin A supplementation. Retinol also reaches colonocytes via the circulation either bound to retinol binding protein (RBP), incorporated into chylomicrons, or as free retinol. Once absorbed from the gut, retinol is esterified, forming retinyl esters that are packaged for export to the liver via chylomicrons (14). Serum retinol levels in non-vitamin A deficient animals vary from 1-2 μM, regardless of supplementation status, [for a review please see: (61)]. However, the liver is the main vitamin A storage site and the primary target for colorectal tumor metastases (181). Hepatic retinyl ester levels increase in response to supplementation and values of > 90 μM have been reported (62). Importantly, dietary supplementation with the retinyl ester, retinyl palmitate, was recently shown

to decrease the incidence of preneoplastic aberrant crypt foci in rats administered 1,2-dimethylhydrazine and consuming a high fat diet (34,159). Dietary retinyl palmitate also prevented the high fat diet-induced increase in colonocyte β-catenin protein levels (159).

β-Catenin is a multifunctional protein that plays a crucial role in the development of colorectal cancer (108). β-Catenin has two major functions in a cell: (1) β-catenin is a component of the membrane-bound adherens complex that stabilizes the plasma membrane and (2) nuclear β-catenin acts as an initiator for the transcription factors T cell factor and lymphoid enhancer factor (TCF/LEF) (182). Levels of free β-catenin in the cytosol are regulated by three proteosomal degradation pathways including a glycogen synthase kinase (GSK)-3β/adenomatous polyposis coli (APC)/Axin-mediated pathway, a p53/Siah-1/APC pathway and a RXR-dependent degradation pathway. Mutations in β-catenin degradation pathways, which are present in 70-80% of colorectal tumors (84,89), lead to increased nuclear β-catenin. Nuclear β-catenin induces gene transcription through the TCF/LEF complex and modulates the transcription of genes involved in cell proliferation (e.g. cyclin D1 and c-myc) (77,78) and metastasis (e.g. matrix metalloproteinase-7) (79). Our laboratory previously showed that retinol induces the ubiquitination and proteosomal degradation of β-catenin, decreasing β-catenin protein levels and the transcription of genes related to cell proliferation and metastasis, specifically cyclin D1, c-myc and matrixmetalloproteinase 7 (158,183). The ability of retinol to decrease β-catenin protein was mediated by a RXRα-dependent degradation pathway (183).

The effect of nuclear receptors on β-catenin protein and β-catenin-TCF/LEF transcriptional activity varies with nuclear receptor. In general, nuclear receptors, including RAR, RXR, vitamin D receptors (VDR), androgen receptors and peroxisome proliferator-activated receptor γ (PPARγ), interact with β-catenin to directly or indirectly reduce β-catenin-

TCF/LEF-mediated gene transcription. For example, RAR, RXRα, PPARγ, androgen receptors and VDR directly bind β-catenin, sequestering it away from the TCF/LEF complex, thereby decreasing β-catenin-TCF/LEF-mediated gene transcription. In contrast, RXR, PPARγ and VDR are also capable of binding to and shuttling β-catenin out of the nucleus and relocating it to the membrane or transporting β-catenin to the proteasome for degradation. For example, RXRα and PPARγ have been shown to directly interact with β-catenin leading to a decrease in TCF/LEF transcriptional activity in malignant prostate cells (110). RXR also indirectly decreases β-catenin-mediated gene transcription by inducing the proteasomal degradation of β-catenin in several cell lines including APC-null and p53-mutant colon cancer cell lines (96). The data suggests that interaction between nuclear receptors and β-catenin reduces β-catenin-TCF/LEF-mediated gene transcription, ultimately altering cell cycle progression and decreasing cell growth.

Previously, Xiao et. al. showed that a RXR agonist induced β-catenin degradation via a novel RXR-mediated degradation pathway (96). The RXR agonist decreased both β-catenin and RXRα proteins independent of the GSK-3β/APC and p53/Siah-1 degradation pathways. As mentioned previously, our studies showed that retinol, which is not a RXR ligand, increases the proteosomal degradation of β-catenin via a RXR-mediated pathway, but the retinol-induced activation of the RXR pathway occurred independent of a RXR agonist (183). We also demonstrated that the RXR antagonist, PA452, and the knockdown of RXRα, using RXRα small interfering RNA (siRNA), inhibited the ability of retinol to decrease total cellular β-catenin levels in both cell lines, suggesting RXRα facilitates the ability of retinol to decrease β-catenin protein levels (183). Xiao et. al. also showed that RXRα and β-catenin directly interact in the absence of a ligand and removing the AF-1 and DNA binding domain (DBD) regions of RXRα eliminated the RXR-mediated β-catenin degradation (96). RXRα also contains a putative nuclear

export signal in its carboxyl-terminal region (179) and interestingly, RXR ligands suppress RXRα's nuclear export activity (179), supporting our previous finding that the degradation of β-catenin is not induced by the RXR ligand, 9-*cis*-RA.

The objective of the present study was to determine if retinol affected β-catenin and RXRα subcellular localization and binding in ATRA-resistant human colon cancer cell lines. Two ATRA-resistant human colon cancer cell lines were used in this study: the HCT-116 cell line which expresses wild type (wt) p53 and APC, but was heterozygous for phosphorylation-resistant β-catenin (deletion of codon 45: CTNNB1$^{WT/\Delta45}$) (118) and the SW620 cell line which expresses wt β-catenin, mutant p53 (R273H), and was APC null (119). Here we show that retinol increases migration of β-catenin and RXRα from the nucleus into the cytosol concomitant with the β-catenin-RXRα binding. We also demonstrate that cytosolic RXRα is proteasomally degraded and importantly, and show that the RXRα and β-catenin binding is required for the proteosomal degradation of β-catenin.

MATERIALS AND METHODS

Tissue Culture

The ATRA-resistant HCT-116 and SW620 human colon cancer cell lines were obtained from and cultured as recommended by the American Type Culture Collection (Manassas, VA). Specifically, HCT-116 and SW620 cells were cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics (1000 U/ml penicillin and 1000 μg/ml streptomycin). For all experiments, cells were plated in 60 mm culture dishes unless otherwise specified. The following day the medium was removed and replaced with media containing retinoids. All retinoids were prepared as 10 mM stock solutions in 100% ethanol. All treatments, including

control, received equal volumes of the ethanol vehicle. All retinoid manipulations were performed under subdued lighting. Each experiment was repeated three times

Subcellular Fractionization

To determine if retinol altered β-catenin and RXRα protein levels in the cytosol or the nucleus, HCT-116 and SW620 cells were plated in 100 mm dishes at a density of 1.6 x 10^6 cells per plate. The following day the medium was removed and replaced with medium containing 0 or 10 μM retinol. Cells were collected 8, 16, 24 and 48 h after treatment. Cells were scraped into lysis buffer (10 mM HEPES pH .8, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM sodium pyrophosphate, 1 mg/ml leupeptin, 1 mM DTT, 2 mM $NaOV_4$, 1 mg/ml PMSF, 1 mg/ml trypsin inhibitor, and 10 mM aprotinin) and 10% NP-40 was added prior to incubation on ice for 15 min. Next, cells were centrifuged at 12,000 x *g* for 2 min at 4ºC. The supernatant was transferred to a new tube and marked as the cytosolic fraction. The pellet was resuspended in nuclear buffer (50 mM HEPES pH .8, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM sodium pyrophosphate, 1 mg/ml leupeptin, 1 mM DTT, 2 mM $NaOV_4$, 1 mg/ml PMSF, 1 mg/ml trypsin inhibitor, 10 mM aprotinin, and 10% glycerol) and placed on a rocker at 4ºC for 30 min to lyse the nuclei. Next, tubes were centrifuged at 12,000 x *g* for 10 min at 4ºC, yielding the nuclear fraction in the supernatant.

The samples were quantitated and electrophoresed as described previously (183). Membranes were blocked with 5% milk in TBST (10 mM Tris, pH 8, 150 mM NaCl, and 0.5% Tween-20) for 1 h, probed with β-catenin antibody (Transduction Laboratories, catalogue #610157, Beckton Dickinson, CA) at a 1:5000 dilution and RXRα antibody (Santa Cruz Biotechnology, D-20 catalogue #sc-553, Santa Cruz, CA) at a 1:1000 dilution in TBST for 2 h.

After incubation with the corresponding secondary antibody at a dilution of $1x10^4$, immunoreactivity was detected as previously described (183). Membranes were then striped and probed for β-tubulin (BD Pharmigen, catalogue #556321, San Jose, CA) as a cytosolic marker and PARP (Santa Cruz Biotechnology, H-250 catalogue #sc-7150, Santa Cruz, CA) as a nuclear marker. Densitometry was performed using a BioRad Gel Documentation System (Hercules, CA). Results are shown relative to vehicle control and corrected for loading with β-tubulin for the cytosolic marker and PARP for the nuclear marker.

Immunocytochemistry

To determine if retinol increased membrane-associated β-catenin, HCT-116 and SW620 cells were plated on 12 mm cover slips at a density of 8×10^5 cells per plate in 60 mm dishes. The following day the medium was removed and replaced with medium containing 0 or 10 μM retinol. Twenty four h after treatment, cells were fixed on the cover slips using 4% paraformaldehyde for 30 min at room temperature. Cells were then permeabilized with 1% Triton-X in PBS for 10 min at room temperature. Before adding the primary antibody, the cells were washed with 0.02% Tween 20 in PBS for 5 min and then with 0.02% Tween 20/1% BSA in PBS for 5 min. The primary antibody, β-catenin, was diluted as recommended by the manufacturer in 3% BSA in PBS. Cells were incubated for 1 h at 37ºC with β-catenin antibody and washed for 5 min with 0.02% Tween 20/1% BSA in PBS. A fluorescein conjugatated secondary antibody (Santa Cruz Biotechnology, FITC catalogue #sc-2010, Santa Cruz, CA) was diluted as recommended by the manufacturer in 3% BSA in PBS and was bound at 37ºC for 1 hr. After incubation with the secondary antibody, cells were washed with 0.02% Tween 20 in PBS for 5 min and then PBS for 5 min. Negative control slides were probed with the secondary antibody alone. Cells were dried for 1 h at 37ºC. To mount the slides, a mixture of 50% glycerol

in PBS and 2.5% Dabco Quencher (Lancaster, Pelham, NH) was used. Cells were visualized on a fluorescent microscope and photographed.

Immunoprecipitation

To examine the effect of retinol on β-catenin and RXRα protein interaction, HCT-116 and SW620 cells were plated at a density of 8 x 10^5 cells per plate in 60 mm dishes. The following day the medium was removed and replaced with medium containing 0 or 10 μM retinol. Cells were collected 8 and 24 h after treatment. Immunoprecipitation of β-catenin and western blot analysis was conducted as described previously (183). Membranes were probed for RXRα and then striped and probed for β-catenin to control for loading differences. Densitometry was performed using a BioRad Gel Documentation System. Results are shown relative to vehicle control and corrected for loading with total β-catenin.

Quantitative Real-Time RT-PCR Analysis

Quantitative real-time RT-PCR was performed to determine the effect of retinol on RXRα mRNA levels. HCT-116 and SW620 cells were treated with 0 or 10 μM retinol for 8, 16, 24 and 48 h. RNA was analyzed as described previously (183). Briefly, 2 μg of RNA were used to produce cDNA (Promega, Madison, WI). Quantitative real time RT-PCR was performed with SYBR Green dye (Perkin-Elmer-Applied Biosystems, Foster City, CA) as described previously (183). Relative amounts of RXRα cDNA were analyzed using the ΔΔCt method. Primers for RXRα and the internal loading control gene, GAPDH, were as follows: RXRα forward primer 5'-GCG CCA TCG TCC TCT TTA AC-3', RXRα reverse primer 5'-TCT GGG TAC TTG TGC TTG CAG TA-3', generating a 118-bp product (184); GAPDH forward primer: 5'-GCT CAG ACA CCA TGG GGA AGG TG-3', GAPDH reverse primer 5'-CAG CGC CAG CAT CGC CCC ACT TG resulting in a 87-bp product (163). All PCR products were sequenced to confirm

their identity. The quantitative RT-PCR results are shown relative to vehicle control and corrected for GAPDH levels.

Western Immunoblot Analysis

To determine if retinol treatment alters RXRα protein levels, cells were plated as specified above and treated with 0 or 10 μM retinol for 48 and 72 h. RXRα western blot analysis was performed as described previously (183). To determine if the decrease in RXRα protein after 48 h of retinol treatment was due to proteasomal degradation, both cell lines were plated as specified above and treated with 0 or 10 μM retinol for 48 h with and without 1 μM of the proteasomal inhibitor MG132 (BIOMOL Research Labs, Plymouth Meeting, PA) for the last 24 h as described previously (183) and subjected to western blot analysis for RXRα.

Construction and Transfection of the RXRa-DBD plasmid

The full-length cDNA encoding RXRα was generously donated by Dr. Ron Evans (Salk Institute, La Jolla, CA) and ligated into the mammalian expression vector pCMX (185). The RXRα-DBD construct, which refers to RXRα without the AF-1 and DBD regions, was generated by cutting the plasmid containing full-length RXRα with restriction enzymes PstI and SalI to eliminate the AF-1 and DBD regions of RXRα [for the RXRα sequence and restriction enzyme digest sites please see (185)]. The resulting plasmid is referred to as pCMX RXRα-DBD. To determine if β-catenin is bound to RXRα in the AF-1/DBD, as previously reported (96,186), cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as described previously (183), with 0.5 μg of the empty vector, pCMX or pCMX containing RXRα-DBD and treated with retinol for 24 h.

Data Analysis

Statistical analyses were performed using Excel (XP 2002; Microsoft). Two-tailed, paired student's t-tests were performed to determine differences between vehicle control and retinol-treated cells. Data are expressed as mean ± SEM, n=3. Differences were considered significant at $P < 0.05$.

RESULTS

Retinol treatment decreases nuclear β-catenin and RXRα protein levels.

Previously, we showed that retinol treatment reduced total β-catenin protein levels and β-catenin-mediated gene transcription, leading us to hypothesize that retinol decreased nuclear β-catenin levels (183). To determine the effect of retinol treatment on nuclear β-catenin and RXRα protein levels, subcellular fractionization was performed on HCT-116 and SW620 cells. A decrease in nuclear β-catenin was seen as early as 8 h after retinol treatment in the HCT-116 cell line (Figure 4.1A). After 24 h of retinol treatment, HCT-116 cells displayed both a significant increase in cytosolic β-catenin and a decrease in nuclear β-catenin (Figure 4.1C). SW620 cells also tended to decrease nuclear β-catenin ($P = 0.10$) as early as 8 h after retinol treatment (Figure 4.1B). This trend continues through 24 h of retinol treatment inducing increased cytosolic β-catenin ($P = 0.09$) and decreased nuclear β-catenin ($P = 0.10$) (Figure 4.1D). To ensure the cytosolic and nuclear fractions were pure, western blot analysis was performed on total cytosolic or nuclear fractions probing for β-tubulin and PARP, respectively, prior to immunoprecipitation (data not shown). Taken together, these data are consistent with retinol increasing β-catenin-RXRα protein binding as early as 8 h after retinol treatment.

To examine the effect of retinol treatment on membrane-bound β-catenin, immunocytochemistry of β-catenin was performed on HCT-116 cells. Immunocytochemistry showed that β-catenin appears to increase at the cell membrane in response to retinol treatment in

the HCT-116 cell line (Figure 4.1E vs F). Photographs of SW620 cells are not shown because these cells exhibit a rounded morphology, preventing a distinct plane of focus.

RXRα protein levels demonstrated a change in subcellular distribution similar to β-catenin in response to retinol treatment. Specifically, RXRα protein levels significantly decreased in the nuclei of both HCT-116 and SW620 cells as early as 8 h after retinol treatment (Figure 4.2 A and B). This significant decrease in nuclear RXRα protein continued through 24 h of retinol treatment in both the HCT-116 and SW620 cell lines (Figure 4.2 C and D). The HCT-116 cells also exhibited a significant increase in cytosolic RXRα following 8 and 24 h of retinol treatment (Figure 4.2 A and C). Taken together, these data suggest that retinol treatment results in decreased nuclear and increased cytosolic β-catenin and RXRα. Retinol also causes increases in membrane-associated β-catenin in the HCT-116 cell line. The movement of β-catenin out of the nucleus is associated with the decreased transcription of the cell proliferation and metastasis associated genes observed previously (183).

Retinol increases β-catenin and RXRα binding.

Because RXRα and β-catenin appeared to partition to the same subcellular fractions in response to retinol treatment, β-catenin protein was immunoprecipitated from total cell lysates to determine the effect of retinol on β-catenin and RXRα protein interaction in HCT-116 and SW620 cells. After only 8 h, both cell lines showed a significant increase in β-catenin and RXRα binding in response to retinol treatment (Figure 4.3 A and B). This increase in β-catenin-RXRα binding continued through 24 h of retinol treatment (data not shown).

β-Catenin protein was also immunoprecipitated from the cytosolic and nuclear fractions to determine if these two proteins were bound in these subcellular components following retinol treatment. After only 8 h of retinol treatment, both cell lines showed a significant increase in β-

catenin and RXRα binding in the cytosol and nucleus (Figure 4.3 C and D). This increase in β-catenin-RXRα binding in the nucleus was exhibited after 24 h of retinol treatment as well (data not shown).

Retinol increases RXRα mRNA levels.

Previously, we showed that retinol treatment increased total cellular RXRα protein levels after 24 h of treatment (183). In the current study we used quantitative real-time RT-PCR to determine if retinol treatment alters RXRα mRNA levels. After 8 h of 10 μM retinol treatment, the SW620 cell line showed a significant increase in RXRα mRNA levels when compared to control (Figure 4.4B). The HCT-116 cell line did not exhibit a significant increase in RXRα mRNA levels until 16 h of retinol treatment (Figure 4.4A). Both cell lines continue to show elevated RXRα mRNA levels through 24 h of retinol treatment. Retinol treatment did not continue to increase RXRα mRNA levels after 48 h (Figure 4.4 A and B) and significantly decreased RXRα mRNA levels in the SW620 cell line (Figure 4.4B).

To determine if the increase in RXRα protein was caused by an increase in protein translation, HCT-116 and SW620 cells were treated with 0 and 10 μM retinol with and without 10 μg/ml cyclohexamide, an inhibitor of protein translation, for 24 and 48 h. Treatment with cyclohexamide failed to block the ability of retinol to increase RXRα protein levels (data not shown). To evaluate if retinol extended the half-life of RXRα mRNA, cells were treated with 0 and 10 μM retinol with and without 2 μg/ml actinomycin D for 8, 16 and 24 h. Treatment with actinomycin D eliminated the retinol-induced increase in RXRα mRNA levels, suggesting that retinol triggers an increase in RXRα transcription (data not shown). These data show that retinol treatment increases RXRα mRNA levels as early as 8 h after retinol treatment in the SW620 cells and after 16 h of retinol treatment in the HCT-116 cells, resulting in the increase in RXRα

protein levels in response to 24 h of retinol treatment observed previously (183), indicating that retinol induces the transcription of the RXRα gene.

Retinol increases the proteasomal degradation of RXRα protein at 48 h.

Retinol treatment increases RXRα mRNA levels through 24 h but not 48 h (Figure 4.4) resulting in the elevated RXRα protein levels observed previously (183), and contributing to the increase in β-catenin-RXRα binding due to retinol treatment (Figure 4.3). Because RXRα is bound to β-catenin and β-catenin protein levels decrease in response to retinol treatment between 24 and 48 h (183), we examined the effect of retinol on total RXRα protein levels at 48 h. We found that after 48 h of retinol treatment, RXRα protein levels are significantly reduced in both HCT-116 and SW620 cells when compared to vehicle control treated cells (Figure 4.5 A and B).

To determine if the decrease in total RXRα protein in response to 48 h of retinol treatment was due an increase in the proteasomal degradation of RXRα, HCT-116 and SW620 cells were treated with the proteasomal inhibitor, MG132. MG132 is a reversible, selective aldehdye inhibitor of cysteine and serine proteases that enters cells rapidly and reversibly (171). Treatment with MG132 blocked the retinol-induced decrease in RXRα protein in both cell lines indicating that retinol decreases RXRα via proteasomal degradation following 48 h of retinol treatment (Figure 4.5 C and D). For example, after 48 h, HCT-116 and SW620 cells treated with 10 μM retinol and 1 μM MG132 showed a significant increase in RXRα protein levels when compared to cells treated with 10 μM retinol alone (Figure 4.5 C and D). We previously demonstrated that MG132 treatment also blocks the ability of retinol to decrease β-catenin (183). Taken together, these data suggest that retinol increases RXRα mRNA and protein levels by 24 h then, after binding with β-catenin, both RXRα and β-catenin are proteasomally degraded, resulting in the decrease in RXRα protein observed following 48 h of retinol treatment.

RXRα and β-catenin binding are essential for the degradation of β-catenin in response to retinol treatment.

The AF-1 and DBD of RXRα are the regions of the RXRα that bind β-catenin (96,186). To determine if β-catenin and RXRα binding is required for the retinol-induced decrease in β-catenin, cells were transfected with an empty vector, pCMX, or pCMX RXRα-DBD and treated with 0 and 10 μM retinol for 24 h. Transfection of cells with pCMX RXRα-DBD eliminated the retinol-induced decrease in β-catenin protein when compared to vector-transfected control cells (Figure 4.6). These data suggest that the increase in β-catenin and RXRα binding in response to retinol is essential for the RXR-mediated proteasomal degradation of β-catenin protein.

DISCUSSION

Here we show that retinol induces RXRα and β-catenin binding and their transport to the cytosol where both proteins are proteasomally degraded in two ATRA-resistant human colon cancer cell lines. The ATRA-resistant human colon cancer cell lines we used each contained a mutation in a β-catenin degradation pathway. The HCT-116 cell line was heterozygous for phosphorylation-resistant β-catenin (deletion of codon 45: $CTNNB1^{WT/\Delta 45}$) (118) and the SW620 cell line has mutant p53 (R273H), and was APC null (119). In a previous study, we demonstrated that retinol decreased total β-catenin protein levels in these ATRA-resistant human colon cancer cells by increasing the ubiquitin-mediated proteasomal degradation of β-catenin, leading to a decrease in TCF/LEF-mediated gene transcription, lowering levels of cyclin D1 and c-myc (183). The ability of RXRα to decrease β-catenin protein was dependent on the RXRα-mediated degradation pathway and was observed regardless of mutation (183).

In the current study, retinol treatment triggered an increase in cytosolic but a decrease in nuclear β-catenin and RXRα starting at 8 h in both cell lines (Figure 4.1 and 4.2). Retinol

treatment also induced RXRα-β-catenin binding as early as 8 h after retinol treatment (Figure 4.3). After 24 h of retinol treatment, RXRα protein was amplified (183) due to increased RXRα mRNA levels (Figure 4.4), but after 48 h of retinol treatment, RXRα protein levels were significantly decreased (Figure 4.5). Treatment with a proteasomal inhibitor blocked the decrease in RXRα protein at 48 h (Figure 4.5). Also, removing the AF-1 and DBD regions of RXRα eliminated the ability of retinol to decrease β-catenin protein (Figure 4.6). Therefore, we hypothesize that retinol induces RXRα and β-catenin binding and their transport to the cytosol where both proteins are proteasomally degraded**,** inhibiting β-catenin-mediated gene transcription and, ultimately resulting in decreased expression of genes involved in cell proliferation (Figure 4.7).

To our knowledge, the effects of retinol on β-catenin *in vitro* have only been examined in our laboratory, however some *in vivo* studies point to a link between dietary vitamin A, colon cancer and β-catenin. For example, a study by Delage et al (34) showed that dietary supplementation with retinyl palmitate reduced the occurrence of carcinogen and high fat diet-induced aberrant crypt foci. Because retinyl palmitate is converted to retinol in the intestinal lumen, the colonocytes of these rats were exposed to retinol. Supplementation with retinyl palmitate also prevented the increase in colonocyte β-catenin due to consumption of a high fat diet (159), indicating that retinol may decrease β-catenin protein levels *in vivo* as well as *in vitro*. In human patients, cellular retinol binding protein (CRBP)-I levels were decreased in hepatocellular carcinomas (172). CRBP I is expressed ubiquitously and facilitates retinol uptake into the target cell and directs the intracellular metabolism of retinol (14,17,18). Reduced CRBP-I levels were also associated with a lower hepatocellular carcinoma two-year survival rate (172). Interestingly, nuclear CRBP-I inclusions were co-localized with nuclear β-catenin in

hepatocellular carcinomas, indicating a potential cross-talk between β-catenin and CRBP-I, and potentially retinol (172).

Previous studies have shown that RXRα interacts with β-catenin and that this interaction is enhanced by, but does not require the presence of, a synthetic RXR agonist (96,110). RXRα also contains a putative nuclear export signal in its carboxyl-terminal region (179) and RXR ligands suppress RXRα's nuclear export activity (179). RXR agonists did not mimic the ability of retinol to decrease β-catenin protein levels in ATRA-resistant colon cancer cell lines (183), suggesting the RXRα-mediated degradation pathway is active in the absence of a ligand. Our previous study also showed that retinol, which is not a RXR ligand, increases RXRα protein levels after 24 h of treatment, concomitant with a reduction in β-catenin protein levels (183). We also demonstrated that the RXR antagonist, PA452, and the knockdown of RXRα, using RXRα siRNA, inhibited the ability of retinol to decrease total cellular β-catenin levels in both cell lines, suggesting RXRα is essential for retinol to decrease β-catenin protein (183).

Xiao, et al. showed that removing the AF-1 and DBD regions of RXRα eliminated the RXRα-mediated degradation of β-catenin (96). In the current study, we further demonstrate that the RXRα-mediated β-catenin degradation pathway is responsible for the retinol-induced decrease in β-catenin protein in ATRA-resistant colon cancer cell lines. Specifically, retinol induces β-catenin-RXRα binding (Figure 4.3) and transport of these proteins from the nucleus to the cytosol (Figure 4.1 and 4.2). Removal of the AF-1 and DBD region of RXRα eliminated the ability of retinol to decrease β-catenin protein (Figure 4.6). Taken together, these data suggest that β-catenin binds directly to RXRα and the co-localization of β-catenin and RXRα to the cytosol and the subsequent ubiquitination of β-catenin observed previously (96), facilitates β-catenin degradation in response to retinol treatment.

RXRα and β-catenin proteins migrate to the cytosol (Figure 4.1 and 4.2) and RXRα protein is then decreased after 48 h of retinol treatment (Figure 4.5). Treatment with MG132 blocked the decrease in RXRα protein at 48 h (Figure 4.5) and we previously showed that MG132 also prevented the retinol-induced decrease in β-catenin protein (183), indicating the bound β-catenin and RXRα proteins are both proteasomally degraded. RXRα protein did not show an increase in ubiquitination before degradation (data not shown). We believe the lack of ubiquitination on RXRα may be because it is bound to β-catenin (Figure 4.3) which is ubiquitinated before proteasomal degradation, targeting both proteins for degradation (183).

The HCT-116 and SW620 cell lines show elevated RXRα mRNA levels in response to retinol treatment (Figure 4.4). Little information is available regarding the regulation of RXRα gene expression. One study shows that RXRα mRNA levels are increased by fatty acids, dexamethasone (a synthetic glucocorticoid), insulin and ATRA, strongly suggesting that lipid and hormonal signaling pathways may regulate RXRα gene expression (187), but a mechanism leading to the observed increase in RXRα mRNA has yet to be determined. The steps directly linking retinol to increased RXRα gene expression will be explored in future studies.

In conclusion, this study shows that retinol treatment induces β-catenin-RXRα binding and their transport to the cytosol, thereby decreasing nuclear β-catenin and RXRα protein levels, in ATRA-resistant human colon cancer cell lines. Once in the cytosol, both β-catenin and RXRα are proteasomally degraded. Importantly, we show that the degradation of β-catenin requires RXRα-β-catenin binding because the ability of retinol to induce the degradation of β-catenin is blocked in cells transfected with a RXRα construct lacking the AF-1 and DBD. We hypothesize that retinol inhibits ATRA-resistant human colon cancer cell growth by decreasing free nuclear β-catenin protein levels via increasing β-catenin-RXRα binding, reducing the transcription of

cyclin D1 and c-myc, ultimately resulting in slowed cell cycle progression. Resistance to ATRA due to loss of RAR expression is a common occurrence during carcinogenesis (30), limiting the effectiveness of ATRA chemotherapy. Because elevated retinol levels can be achieved in the intestinal lumen via dietary vitamin A supplementation, retinol, or a synthetic derivative of it may prove to be a successful colon cancer chemotherapy.

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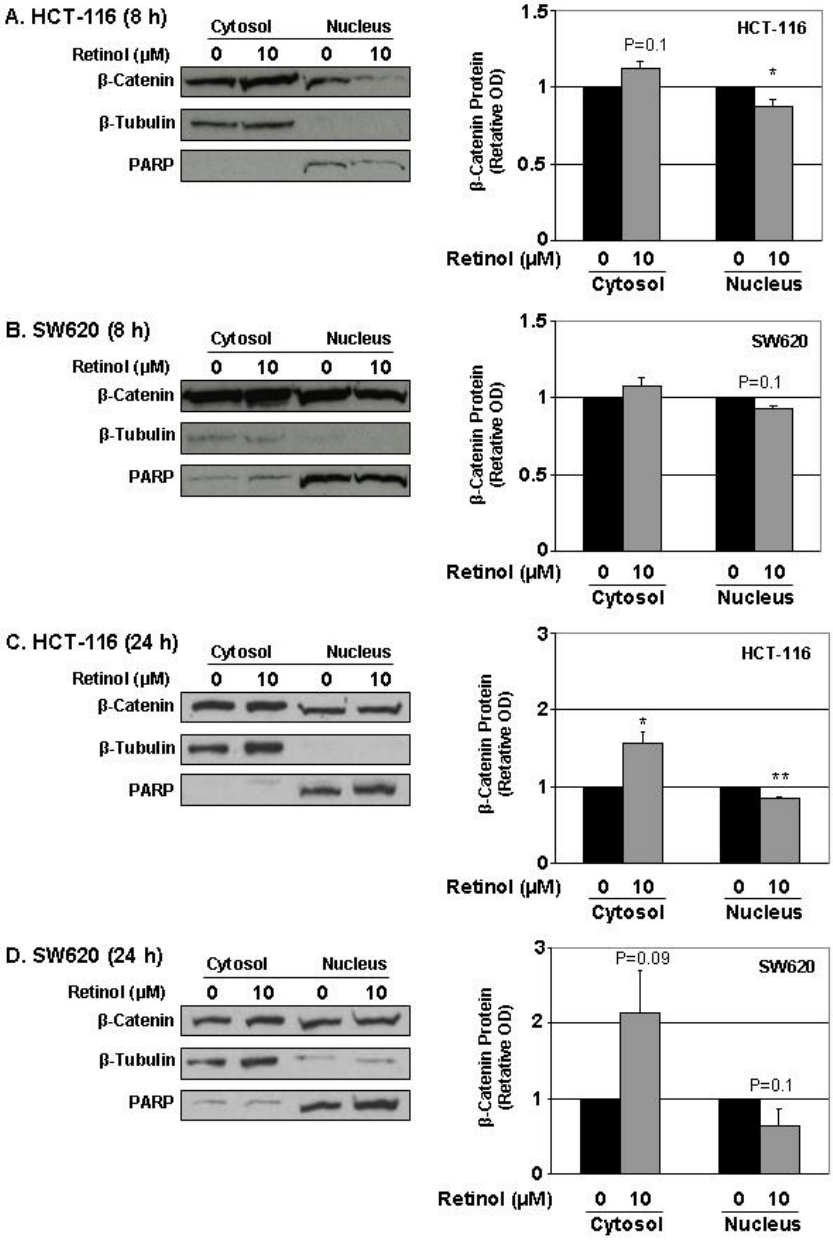
A. HCT-116 (8 h)
Cytosol
Nucleus
Retinol (μM)
0 10 0 10
β-Catenin
β-Tubulin
PARP
β-Catenin Protein (Retative OD)
HCT-116
P=0.1
*
Retinol (μM)
0 10 0 10
Cytosol
Nucleus
B. SW620 (8 h)
SW620
P=0.1
C. HCT-116 (24 h)
**
D. SW620 (24 h)
P=0.09
E. HCT-116 Control (24 h)
F. HCT-116 10 μM Retinol (24 h)

Figure 4.1 Retinol decreases nuclear β-catenin. HCT-116 and SW620 cells were treated with 0 and 10 μM retinol for 8 (A and B) and 24 (C and D) h. After 8 and 24 h of retinol treatment, cells were divided into cytosolic and nuclear fractions. Protein was extracted and immunoblotted for β-catenin, PARP (nuclear fraction marker) and β-tubulin (cytosolic fraction marker). These experiments were performed three times with similar results; one representative western blot is shown. Values shown are the mean of three separate experiments ± SEM. $*P < 0.05$, $**P < 0.01$; significantly different from vehicle control. HCT-116 cells treated with ethanol vehicle (E) or 10 μM retinol (F) were stained with β-catenin antibody and visualized on a fluorescent microscope as described in the materials and methods.

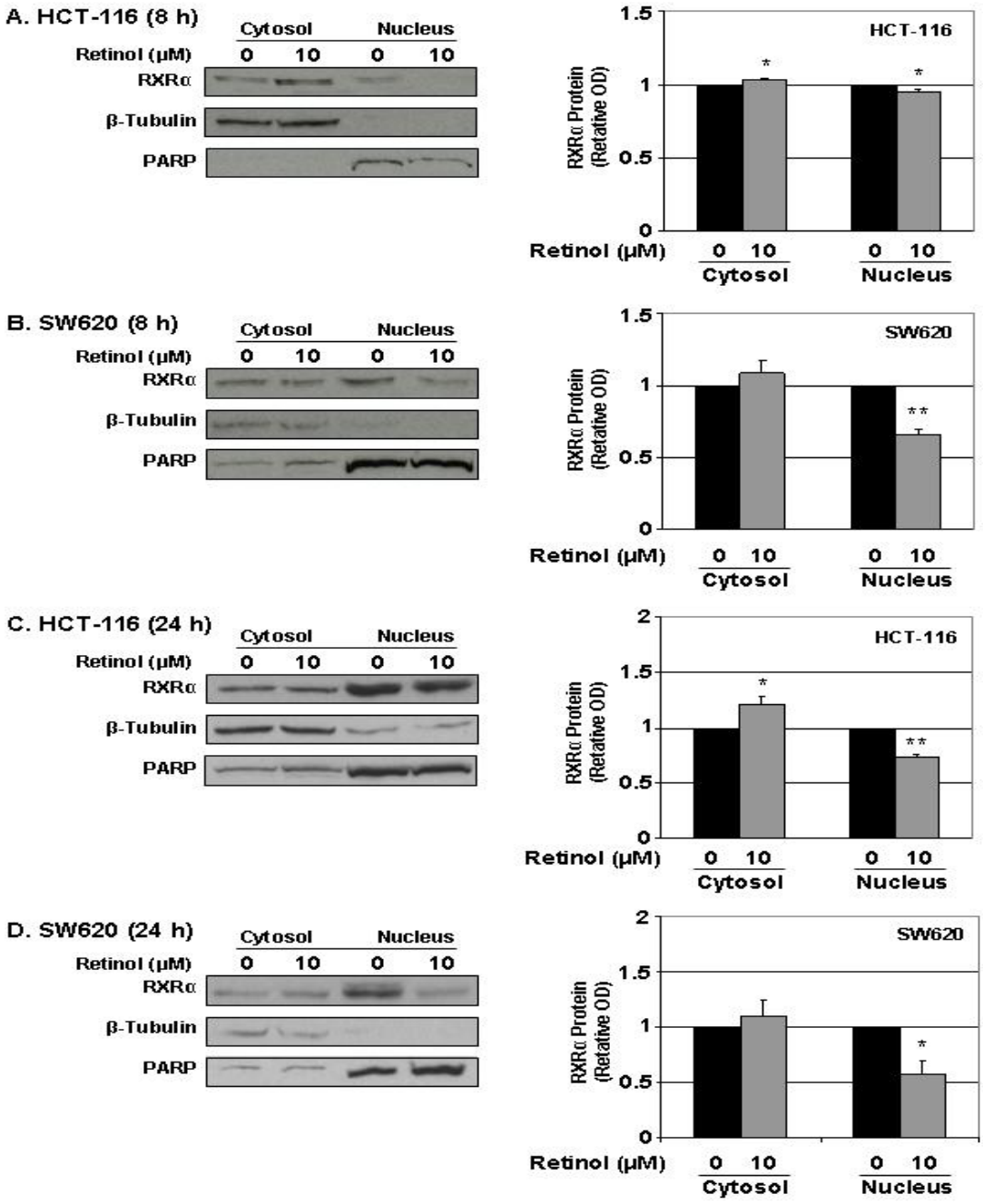

Figure 4.2 Retinol decreases RXRα in the nucleus. HCT-116 and SW620 cells were treated with 0 and 10 μM retinol for 8 (A and B) and 24 (C and D) h. After 8 and 24 h, cells were divided into cytosolic and nuclear fractions. Protein was extracted and immunoblotted for RXRα, PARP (nuclear fraction marker) and β-tubulin (cytosolic fraction marker). This experiment was performed three times with similar results; one representative western blot is shown. Values shown are the mean of three separate experiments ± SEM. *$P < 0.05$, **$P < 0.01$; significantly different from vehicle control.

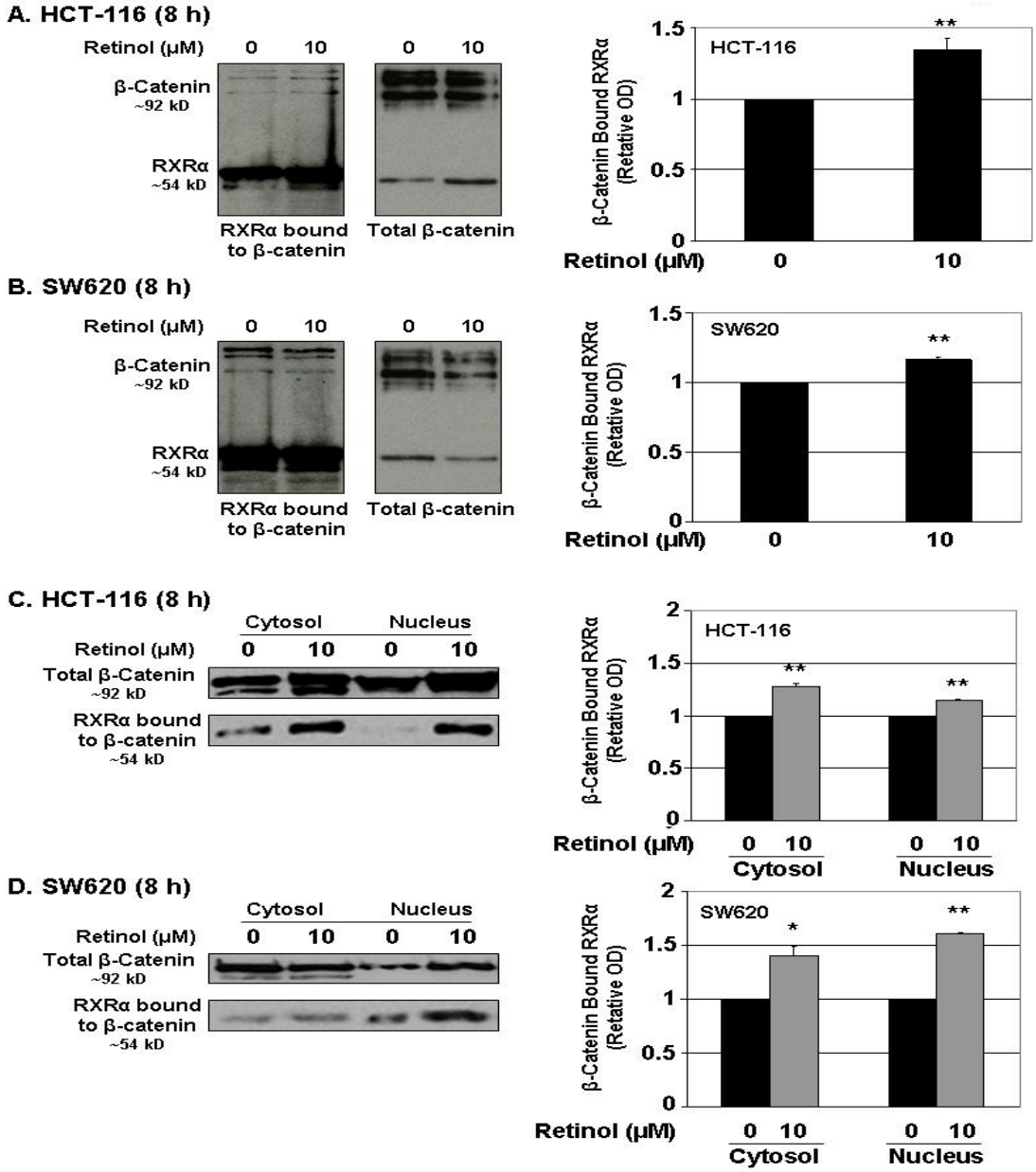

Figure 4.3 Retinol increases β-catenin and RXRα binding. HCT-116 and SW620 cells were treated with 0 and 10 μM retinol. After 8 h of treatment, total protein was harvested, and cell extracts were immunoprecipitated using a β-catenin antibody and immunoblotted for RXRα and β-catenin (A and B). After 8 h of treatment, cells were divided into cytosolic and nuclear fractions, and cell extracts from each fraction were immunoprecipitated using a β-catenin antibody and immunoblotted for RXRα and β-catenin (C and D). These experiments were performed three times with similar results; one representative western blot is shown. β-Catenin-RXRα binding was calculated by dividing total RXRα protein by total β-catenin protein and normalizing to vehicle control. Values shown are the mean of three separate experiments ± SEM. **P* < 0.05, ***P* < 0.01; significantly different from vehicle control.

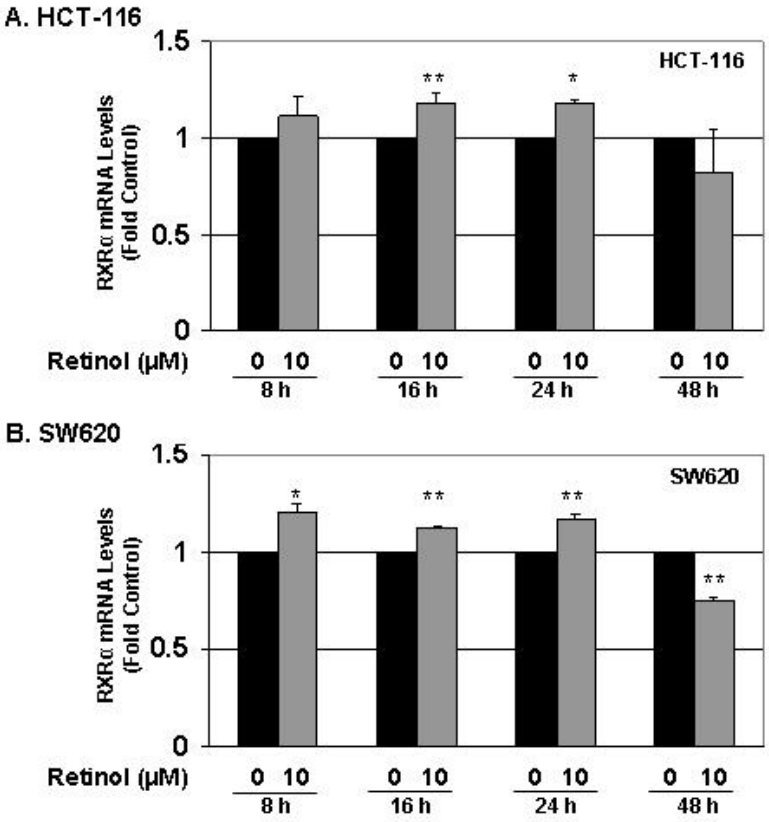

Figure 4.4 Retinol increases RXRα mRNA levels through 24 h. Quantitative real time RT-PCR was performed as described in Materials and Methods. (A) HCT-116 and (B) SW620 cells were treated for 8, 16, 24 and 48 h with 0 and 10 μM retinol. Total RNA was reverse transcribed using random primers and then amplified on the ABI 7900HT machine (Perkin-Elmer-Applied Biosystems, Foster City, CA) using the SYBR green I Quantitect kit and analyzed using the ΔΔCt method. Experiments were repeated three times and each PCR reaction was run in duplicate. RXRα mRNA levels are shown relative to vehicle control and corrected for GAPDH. Values shown are the mean of three separate experiments ± SEM. $*P < 0.05$, $**P < 0.01$; significantly different from vehicle control.

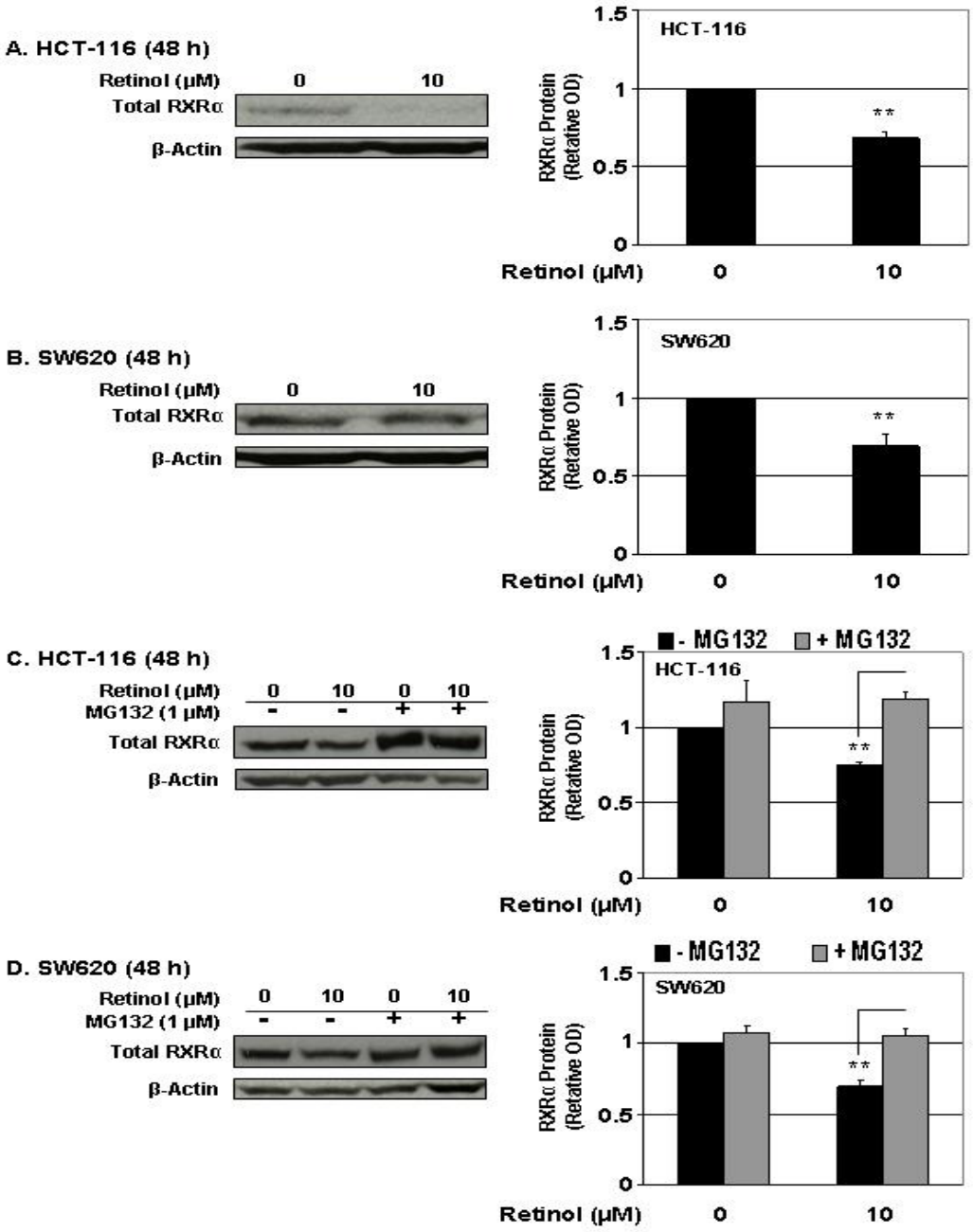

Figure 4.5 Retinol decreases RXRα protein at 48 h through increased proteasomal degradation. Total RXRα protein levels were measured in (A) HCT-116 and (B) SW620 cells treated for 48 h with 0 and 10 μM retinol. Total protein was harvested and immunoblotted for RXRα and β-actin as described (183). (C) HCT-116 and (D) SW620 cells were treated with 0 and 10 μM retinol with and without 1 μM of the proteasomal inhibitor, MG132. After 48 h, total protein was harvested, electrophoresed and probed for RXRα and β-actin. This experiment was performed three times with similar results; one representative western blot is shown. Values shown are the mean of three separate experiments ± SEM. $*P < 0.05$, $**P < 0.01$; significantly different from vehicle control (A and B) or plus MG132 (C and D).

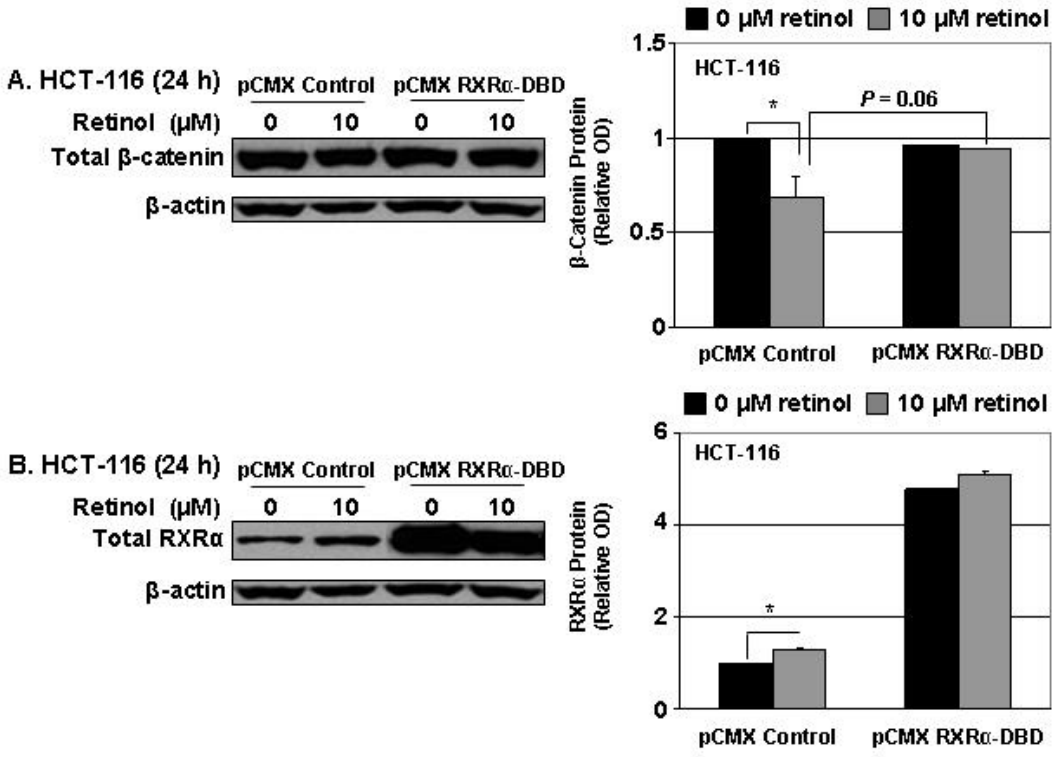

Figure 4.6 RXRα DNA binding domain is necessary to decrease β-catenin protein. HCT-116 cells were transfected with pCMX vector (empty vector control) or pCMX RXRα-DBD and treated with and without retinol for 24 h. Total protein was harvested, electrophoresed and probed for (A) β-catenin and (B) RXRα. This experiment was performed three times with similar results; one representative western blot is shown. Values shown are the mean of three separate experiments ± SEM. *$P < 0.05$; significantly different from vehicle control.

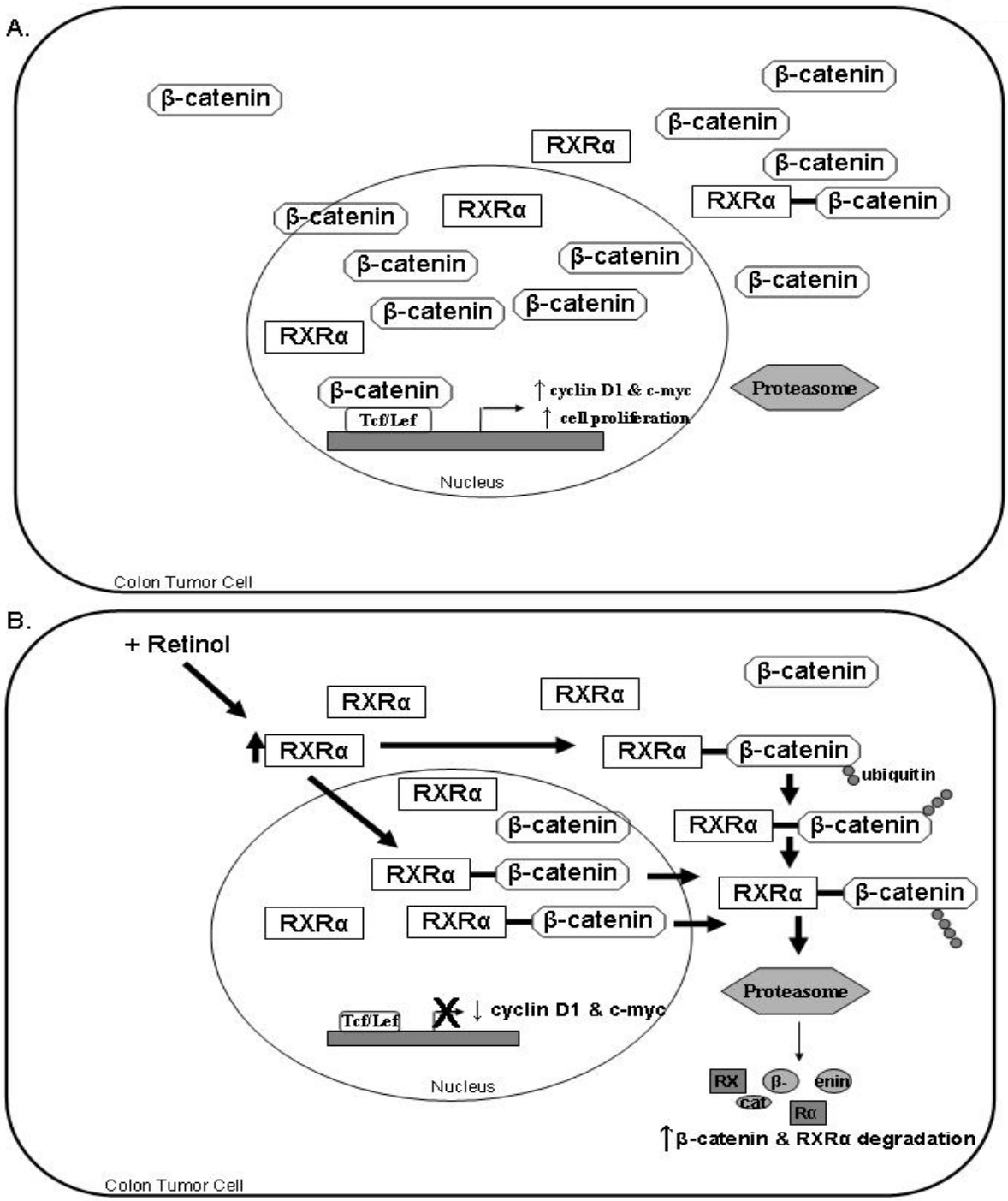

Figure 4.7 Model proposing role of retinol-induced degradation of β-catenin. Retinol treatment for 24 h results in an increase in RXRα-β-catenin binding. The RXRα-β-catenin complex then moves from the nucleus to the cytosol. Once in the cytosol, β-catenin is ubiquitinated and both RXRα and β-catenin are degraded by the proteasome resulting in decreased RXRα and β-catenin levels by 48 h. The decrease in β-catenin due to retinol treatment results in decreased transcription of the β-catenin/TCF/LEF mediated genes cyclin D1 and c-myc (183), resulting in slowed cell cycle progression and decreased cell proliferation (158).

Chapter 5: Summary and Future Directions

Summary

Cardiovascular disease is overall the leading cause of death for Americans of all ages, however among Americans under the age of 85, cancer is the most common cause of death (2). Colorectal cancer is the third leading cause of cancer death in the United States in both men and women. Even with the advances made in understanding and treating cancer, cancer occurrence and survival rates have remained relatively unchanged in the last 20 years (2). Lifestyle, specifically diet, is associated with the majority of all chronic diseases (3) and changes in diet are a relatively easy way to reduce the risk of many diseases. Many studies have looked at different compounds in the diet, including vitamin A, as potential chemotherapies for colorectal cancers with fewer adverse side affects than the current chemotherapy treatments. With their potent antiproliferative effects on cancer cells *in vitro*, retinoids may have potential for chemotherapy of colon cancer. The goal of this dissertation is to evaluate the efficacy of retinol as an inhibitor of ATRA-resistant human colon cancer cell line growth and to determine the mechanism by which retinol decreases β-catenin protein *in vitro*.

Chapter 1 introduced the importance of inhibiting cancer cell growth and retaining command of the cell cycle to ensure controlled cell growth. It explains the importance of proper β-catenin protein regulation as well as the pathways necessary to degrade β-catenin protein. It also provides background information on the structure, absorption and function of vitamin A and the potential for ATRA resistance. The cell lines used in the investigation presented were also reviewed.

Chapter 2 focused on the initial studies showing how retinol inhibits the growth of ATRA-sensitive and -resistant human colon cancer cell lines by slowing cell cycle progression

though a RAR-independent mechanism. First, three ATRA-resistant colon cancer cell lines, HCT-116, SW620, and WiDr cells were growth inhibited with retinol treatment for 96 h. This growth inhibition was caused by a slowed cell cycle progression in each cell line. Retinol did not induce apoptosis, necrosis or cellular differentiation in these cell lines. Retinol was not metabolized into bioactive metabolites and the growth inhibition was not dependent on the traditional ATRA/RAR/RXR/RARE pathway, indicating that retinol is not being metabolized into sufficient amounts of ATRA or another bioactive metabolite to induce RAR-dependent gene transcription. Chapter 3 investigates the ability of retinol to decrease β-catenin protein levels by increasing ubiquitin-mediated proteasomal degradation. First, three ATRA-resistant colon cancer cell lines showed a significant decrease in β-catenin protein when treated with retinol for 24 and 48 h regardless of mutation. This decrease was due to an increase in β-catenin ubiquitination and proteasomal degradation. The retinol-induced decrease in β-catenin led to a decrease in β-catenin/TCF/LEF-mediated gene transcription. Next, it was determined that retinol treatment significantly increased RXRα protein and using an RXR antagonist and RXRα siRNA, we showed that RXRα was essential for the proteasomal degradation of β-catenin protein in all three ATRA-resistant human colon cancer cell lines. Also, a RXR agonist did not mimic retinol's ability to decrease β-catenin protein. We conclude that the RXR-dependent degradation pathway promotes β-catenin degradation independent of RXR-ligand binding.

Chapter 4 reports that in ATRA-resistant human colon cancer cell lines, retinol decreases β-catenin and RXRα in the nucleus, induces β-catenin-RXRα binding and their transport to the cytosol where both proteins are proteasomally degraded. We also show that these actions require the RXRα DBD. As shown in Chapter 3, the degradation of β-catenin and RXRα is regulated by the RXR-mediated degradation pathway, the only fully functional β-catenin degradation pathway

in both cell lines. We hypothesize that retinol inhibits ATRA-resistant human colon cancer cell growth by decreasing free nuclear β-catenin protein levels by increasing β-catenin-RXRα binding, decreasing the transcription of cyclin D1 and c-myc, ultimately resulting in slowed cell cycle progression.

Future Directions

Taken together, these studies suggest that retinol, or a derivative of it, may prove an effective chemotherapy to treat colon cancer. These studies add insight into the complex mechanism of how retinol, and not ATRA, inhibits the growth of ATRA-resistant human colon cancer cell lines. Future studies will focus on the ability of retinol to increase RXRα transcription. For example, we showed that retinol, which is not a RXR ligand, induces RXRα transcription increasing RXRα protein levels concomitant with a reduction in β-catenin protein levels, but the link between retinol and increased RXRα has yet to be determined. As mentioned, RAR/RXR heterodimers bind to RARE and stimulate the transcription of RAR. A similar phenomenon with respect RXR gene transcription remains unknown. Little information is available regarding regulation of RXRα gene transcription. One study shows that RXRα gene expression is increased by fatty acids, synthetic glucocorticoids, insulin and ATRA, which strongly suggests that lipid metabolism and hormonal signaling pathways may regulate RXRα gene expression (187) but no mechanism of increased RXRα gene expression has been found. Furthermore, the promoter of the RXRα gene has not been well characterized. Previous work has shown that the promoter region of RXRα is unusually G + C rich, has 17 putative SP1 transcription factor binding sites and has no TATA or CAAT boxes (188). These characteristics make the RXRα promoter an atypical promoter region and the highly rich G + C region gives the RXRα promoter the characteristic of housekeeping gene promoters (188).

One potential future study would be to determine if RXRα transcription is controlled by RXRα levels, just as RAR/RXR heterodimers binding RARE control RAR transcription (31-33). Plasmids could be constructed containing varying regions of the RXRα promoter (for an example see: Figure 5.1) (188). The promoter regions would be ligated to luciferase (Figure 5.2). After transfection into ATRA-resistant colon cancer cell lines and treatment with retinol, if RXRα levels regulate its own transcription, we expect that the RXRα promoter-luciferase reporter constructs containing the region that controls RXRα transcription to exhibit increased luciferase activity. If a region shows increased activity, we would determine what proteins bind to that specific sequence of the RXRα promoter to elucidate what activates that promoter region.

Most functions of RXR require homo- or heterodimerization with another nuclear receptor. It has been shown that direct repeat 1 recruits RXR/RXR, RAR/RXR and PPAR/RXR partners (189) and that RXR homodimers are capable of binding to PPAR/RXR DNA binding sites (174). Therefore, we also think it is important to determine if the retinol-induced increase in RXRα mRNA levels requires RXRα DNA binding and/or a nuclear receptor binding partner. We would investigate if RXRα is involved with an active transcription site in RXRα with an electrophoretic mobility shift assay. To examine possible RXRα dimerization partners immunoprecipitation of RXRα will be performed and then protein will be electrophoresed through a 2D gel. The proteins that precipitated with RXRα will be cut out of the gel and sequenced to determine possible dimerization partners to RXRα. From these experiments, we will determine if RXRα regulates its own transcription and if heterodimerization with another nuclear receptor is necessary in RXRα mRNA regulation. Clearly, more work is needed to understand the mechanism by which retinol increases RXRα mRNA, but we have helped fill the

gap in knowledge between RXRα and β-catenin degradation which is necessary to continue developing potential chemotherapies for ATRA-resistant human colon cancer.

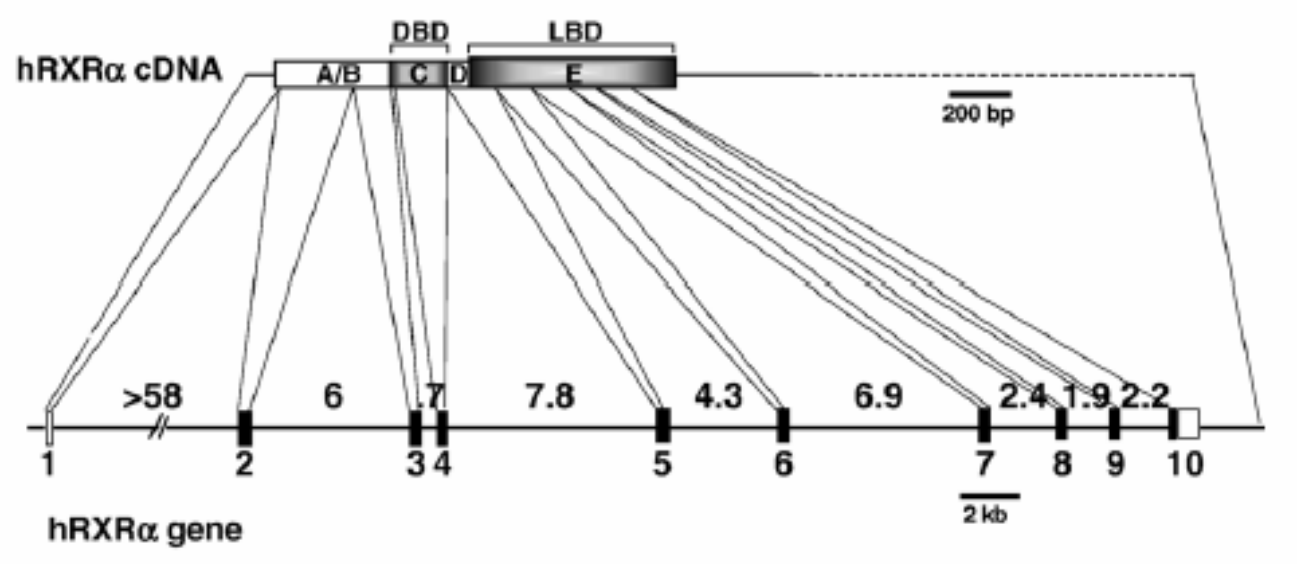

Figure 5.1 Map of the human RXRα gene. The human RXRα cDNA is shown at top. The numbered boxes on the lower diagram represent exons and lines represent introns. Numbers above the line indicate intron size in kilobases (kb). DBD: DNA binding domain; LBD: ligand binding domain; bp: base pairs. From (188).

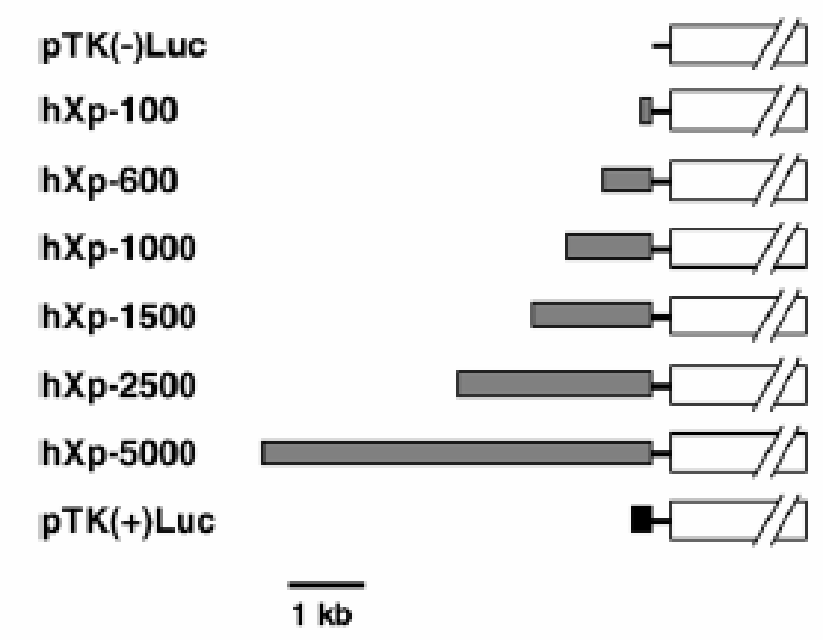

Figure 5.2 Example of RXRα luciferase reporter plasmids. Open white box signifies luciferase protein coding region and the gray boxes signify differing lengths of RXRα promoter (188).

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Vita

Alice Clare Dillard was born in Columbus, Ohio to Jesse and Nancy Dillard. She has two older sisters, Rebecca and Catherine, and one younger brother, James. Alice's family moved to Albuquerque, NM shortly before her 11th birthday in 1991, where she lived until graduating from Eldorado High School in 1998. In the fall of that year she began her college education at the Georgia Institute of Technology. In 2000, Alice chose to pursue an education in Nutritional Sciences and transferred to the University of Georgia. She received her Bachelor of Science in Nutritional Sciences from the University of Georgia in December 2001. She began her graduate work at the University of Texas at Austin in August 2002. She currently resides in Pflugerville, TX.

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